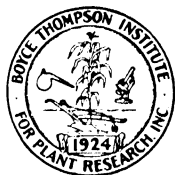




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ERRATA

Page 325, Table II, column 1, for 'var. *perpuscill*' read 'var. *perpuscilla*'

Page 441, Table I, column 5, for '35 (B) —' read '35 (B)'

TOXIC ACTION IN SOIL OF ILLUMINATING GAS CONTAINING HYDROCYANIC ACID

A. E. HITCHCOCK, WILLIAM CROCKER, AND P. W. ZIMMERMAN

INTRODUCTION

It has been known for many years not only that many plants are sensitive to traces of artificial illuminating gas, but that ethylene is the most effective constituent causing certain specific responses such as epinasty, yellowing, and abscission of leaves. This work was reviewed and discussed in a recent paper with particular reference to the epinastic response (4). The conditions under which plants respond to an atmosphere containing illuminating gas are distinctly different in many respects from those involved when illuminating gas is passed through the soil. In the former case extremely low concentrations of illuminating gas (1 part of gas to from 100,000 to 500,000 parts of air) or of ethylene (1 part of gas to 20,000,000 parts of air) are effective, the response of aerial parts is specific, the effective constituents are known, prolonged periods of exposure (usually several days) to low concentrations are necessary to cause noticeable injury, the roots show little or no injury, and the plants recover if transferred to a normal atmosphere of air. High concentrations of illuminating gas cause essentially the same responses in a shorter period of time, and the final amount of injury is much greater.

When illuminating gas is passed through the soil, relatively large amounts (from one cubic foot to several thousand cubic feet) are required to cause injury, the responses are usually non-specific, all of the effective constituents are not known, severe injury may result from short periods of exposure (from less than one hour to several hours), the roots always show injury regardless of the effect on aerial parts, and the plants recover slowly or not at all after the flow of gas has been stopped, depending upon the amount of toxic residues remaining in contact with the roots.

The possibility that illuminating gas acts as any inert gas, such as nitrogen, in displacing oxygen from the soil has been a view supported by Ehrenburg and Schultze (7) and Sorauer (15). Harvey and Rose (8) considered the displacing effect of illuminating gas important only when there was a rapid flow of undiluted gas. With a relatively slow flow of diluted illuminating gas they considered as the chief cause of injury to roots those constituents which remained in a *gaseous state* in the soil interstices; any injury to the aerial parts was due to lack of water and not to the conduction of toxic substances to the tops as proposed by Stone (16). Wehmer (17) found no difference in the response of cress to illu-

minating gas containing amounts of air ranging from 10 to 95 per cent.

Stone (16), Wehmer (18, 19), and Haselhoff (9) are opposed to the idea that gaseous constituents of illuminating gas held in the soil interstices are mainly responsible for the injury to roots. These three investigators believe that injury to roots is due principally to the condensed products of illuminating gas which remain in the soil. They differ as to whether the injury to aerial parts is a direct or indirect action. Stone (16) considers that toxic materials are transported from the roots to the aerial parts. Wehmer (18) and Haselhoff (9) contend that the injury to aerial parts is an indirect action, being due to disturbed water and food relations resulting from the initial injury to roots.

Wehmer (20, 21) is of the opinion that the hydrocyanic acid in illuminating gas is largely responsible for the injuries to plants. Haselhoff (9) agrees that hydrocyanic acid is injurious but doubts that the small amount normally present in illuminating gas can account for severe injury to plants. Since Harvey and Rose (8) were able to produce with ethylene the same type of abnormalities that occurred as a result of passing illuminating gas through the soil, they believe ethylene to be the principal injurious constituent. The differences of opinion thus far mentioned may be due to the fact that different workers were not dealing with the same type of injury, and the illuminating gases used were probably never identical with respect to kinds and percentages of the constituents. As a result of passing illuminating gas through soil, many different types of responses have been described, such as wilting, drying, curling, discoloration, stunting, epinasty, and abscission of leaves; stunting, swelling, discoloration, proliferation, stimulation, bending, and killing of roots; and proliferation, cracking (sometimes accompanied by exudation of sap), and partial or complete killing of stems, trunks, or branches.

In 1930 experiments were undertaken to determine which constituents in illuminating gas caused certain types of injuries when the gas was passed through soil. A brief report of this work was made in 1932 (11). The purpose of the present paper is to describe more fully these and later experiments. Evidence is presented to show that roots are injured by the residual products from illuminating gas which remain in the soil, as well as by the flowing gas; that when used in equivalent amounts carbon monoxide or the four principal unsaturated hydrocarbon constituents of illuminating gas (ethylene, propylene, butylene, and acetylene) do not produce an equal amount or a similar type of injury; that the initial injury occurs on the roots regardless of whether the aerial parts are affected; that solutions which remove hydrocyanic acid from illuminating gas eliminate most but not all of the highly toxic effects of the gas; and that immediate displacement of vapors in the interstices of gassed soil does not render the soil non-toxic.

MATERIALS

The illuminating gas was taken directly from the mains of the Westchester Lighting Company which supplies gas to the City of Yonkers, and is the same as that described in previous reports (4, 10). Unsaturated hydrocarbon gases were the same as those for which analyses are given in an earlier paper (4, p. 194). The drip oil obtained from a gas company in Brooklyn, New York, contained 88 per cent of "light oil" which had the following constituents according to the analyses furnished by this company: Unsaturated hydrocarbons (mostly indene, styrene, and their homologues) constitute 53.5 per cent, benzol 6.6 per cent, toluol 14.5 per cent, and xylol 25.4 per cent. "Light oil" is the fraction of drip oil which distills below 200°C. Fractional analysis of the drip oil showed that a considerable amount of naphthalene was present. Approximately 147 cubic feet of illuminating gas were calculated to produce one cubic centimeter of drip oil.

The principal plants used in these experiments are as follows: Japanese maple (*Acer palmatum* Thunb.), sweet cherry (*Prunus avium* L.), silver-bell (*Ilaesia carolina* L.), persimmon (*Diospyros virginiana* L.), rose (*Rosa*, hybrid tea var. Coolidge), privet (*Ligustrum ovalifolium* Hassk.), weeping willow (*Salix babylonica* L.), cotton (*Gossypium hirsutum* L.), tung oil (*Aleurites* sp.), Jerusalem cherry (*Solanum pseudocapsicum* L.), mallow (*Hibiscus moscheutos* L.), sunflower (*Helianthus debilis* Nutt.), tomato (*Lycopersicon esculentum* Mill.), and Boston fern (*Nephrolepis exaltata* Schott. var. *bostoniensis* Davenport).

METHODS

Exposure of roots in soil to flowing gases. Gas was passed in through the hole in the bottom of the pot and allowed to flow up through the soil. In the case of experiments performed in the hood the aerial parts of the plant were sealed in an inverted bell jar. Plants treated outside where there was a free circulation of air were not shielded. Small amounts of gas were measured by a dry meter calibrated to read on one dial to one-eighth of a cubic foot and on a second dial to 0.08 of a cubic foot.

Fifteen per cent sodium hydroxide, 2 N potassium hydroxide, and acidified 10 per cent silver nitrate were used to remove toxic constituents from illuminating gas. When the gas was flowed over the aerial parts of plants in bell jars, injuries from drying and from lack of oxygen were prevented by drawing air into the bell jar through water at the same rate as the illuminating gas when the period of exposure was three hours or longer.

Toxicity of residues from illuminating gas. Plants from pots or flats were transferred to soil through which illuminating gas had been passed. The effect of illuminating gas residues left in the soil was also compared

with the effect produced by exposure of the roots in soil to flowing gas. Thus, plants subjected to flowing gas were transferred immediately after the flow of gas was stopped to control soil and plants from control soil were transferred to the gassed soil. A third set of plants was subjected to flowing gas and then repotted in the same gassed soil. The roots of all plants, therefore, received the same mechanical disturbance.

The loss of toxicity from gassed soil was determined by planting seedlings and seeds in soil which had been stored at different temperatures (room temperature, 3°C ., and -15°C .) in open and in sealed containers. Plantings were also made in soil leached with tap water. The toxicity of the leachings was determined by its effect on seeds of tomato and buckwheat. The rate of loss of toxic vapors from gassed soil was determined by confining samples of the soil under bell jars with young tomato plants.

Addition to soil of compounds present in illuminating gas. Sodium and potassium cyanide, benzol, toluol, xylol, phenol, and drip oil were added separately to soil in amounts sufficient to cause collapse of all underground parts of young tomato seedlings. These toxic soils were then diluted with control soil until the highest dilution produced little or no injury on the tomato plants. Loss of toxicity from these soils was determined in the same manner as described for soil through which illuminating gas had been passed. Toxicity of the vapors from these treated soils was determined the same as for gassed soil.

Chemical methods for detecting illuminating gas in soil. The heavy hydrocarbons and phenols in extracts of gassed soil were determined according to the method of Pfeiffer (12). Direct bromination with a one-tenth saturated solution of bromine was used if the soil extracts were clear. For colored extracts an excess of bromine was added and the excess measured by titrating with sodium thiosulphate in the presence of potassium iodide, using starch as an indicator.

Phenols in extracts from gassed soil were determined qualitatively by a color reaction with an alkaline solution of diazobenzene sulphonic acid. In the presence of small amounts of phenols the dye turns yellow.

Hydrocyanic acid in illuminating gas was determined quantitatively by passing the gas through 15 per cent sodium hydroxide or 2 N potassium hydroxide, and titrating with silver nitrate according to the method of Powell (13), but instead of determining the end point colorimetrically, a silver electrode was used. Due to the development of a dark color in the test solution during titration, the end point was not easily determined by the colorimetric method. The presence of the cyanide radical in illuminating gas was determined qualitatively by means of the Prussian blue test (6, p. 275), the thiocyanate test (6, p. 272), and the copper-benzidine acetate test (5). All three qualitative tests were likewise made on the soil. The Prussian blue and thiocyanate tests required a preliminary treatment of

the soil. Dilute sulphuric acid was added to the soil in a flask and the mixture was heated. The vapors were led into acidified silver nitrate or into sodium hydroxide. Both of these tests were also made on extracts of gassed soil. Gassed soil was also tested by sealing in a small bottle with a strip of filter paper previously dipped in the copper-benzidine acetate solution. Relative amounts of hydrocyanic acid (HCN) and cyanogen (C_2N_2) in the illuminating gas were determined by passage of the gas first through a 10 per cent solution of acidified silver nitrate and then through a 2 N solution of potassium hydroxide (6, p. 272-273).

EXPERIMENTAL RESULTS

EXPOSURE OF ROOTS IN SOIL TO FLOWING GASES

Illuminating gas. The principal responses to a prolonged treatment of roots in soil to illuminating gas were as follows: epinasty, discoloration, stunting, drying, wilting, and abscission of leaves; discoloration, hypertrophy, retarded growth, and the collapse or death of part or all of the stem and roots; the initiation and stimulation of adventitious roots on stems; and the abscission of flowers and fruits or the abnormal retention of fruit. The response of aerial parts was in many cases the same as when the plant was confined with illuminating gas in a tight container. The type and degree of injury produced by flowing illuminating gas depended upon the total volume of gas flowed through the soil, the rate of flow, the volume of soil surrounding the roots, and the species, age, and activity of the plant.

Epinasty was particularly noticeable on the maple, cotton, tung oil, mallow, and tomato; yellowing on the leaves of the tomato and Jerusalem cherry; discoloration other than yellowing on the maple; wilting of leaves on the willow, persimmon, tomato, and sunflower; abscission of leaves on the rose, tomato, and Jerusalem cherry; initiation and stimulation of adventitious roots on the stem of tomato; abscission of flowers and nearly mature fruits from the tomato and Jerusalem cherry, and retention of young fruits on the tomato and Jerusalem cherry.

Discoloration of roots occurred in all cases of injury. The predominating color was brown. Portions of the roots of some species turned pink, particularly in the case of tomato, sunflower, and privet. A pink color usually occurred on roots which were injured but had not been entirely killed at the time the color was observed. Later these roots sometimes died, in which case the pink was frequently replaced by tan, brown, or black. Shriveled tap roots of tomato and sunflower often retained a sufficient amount of color to give them a distinctly pink hue. Roots of tomato sometimes became translucent just previous to their collapse. Roots of maple were frequently bluish-gray or black. Collapse of the stems of young seed-

lings was caused by the exposure of the bare root systems to one cubic foot of flowing illuminating gas. The same amount of ethylene or nitrogen injured some of the bare roots but did not cause collapse of the lower part of the stem.

If root growth was retarded temporarily and there was little or no killing, a renewal of growth from original growing points or the appearance of new branch roots several days after treatment was characterized in some cases by a noticeable increase in diameter, usually for a distance of a few millimeters; thereafter the roots were of a normal diameter.

In the case of certain woody plants, particularly the maple, a slow flow of gas at the rate of one-half cubic foot or less per hour caused complete defoliation in a few days, whereas a flow of gas at the rate of two to eight cubic feet per hour caused slight epinasty and death of the leaves with little or no defoliation. The maximum rate of flow (15 to 20 cubic feet per hour) killed the leaves without causing noticeable epinasty. Several weeks or several months after treatment some of the dead leaves fell from the plant, but frequently they became detached at the point where the petiole joins the leaf blade and not at the point of attachment to the stem as usually occurred when green leaves abscised. When treatment of maple caused epinasty but no immediate drying of leaves, the petioles remained bent for a period of one week or longer, after which they usually died and remained on the plant.

One cubic foot of illuminating gas was approximately the smallest amount which caused severe injury or the death of all roots of tomato plants two to four inches tall grown in four-inch pots. In addition, this amount of illuminating gas frequently caused collapse of the tap root and of that part of the stem which was in soil. Four cubic feet of illuminating gas caused collapse of the roots and lower part of the stem as shown in Figure 1 in all tests and therefore represents an amount well in excess of that necessary to produce minimum injury to young tomato seedlings.

Injuries to the tomato and to five species of woody plants are recorded in Table I. Data in column 3 of Table I show that illuminating gas caused injury to all six species of plants, but that four cubic feet caused greater injury than one cubic foot. The plants in Table I are listed from top to bottom approximately in the order of their decreasing sensitivity to illuminating gas but the same order does not hold for the other gases. All roots of the tomato were killed by four cubic feet of illuminating gas without there being any signs of injury on the aerial parts two to four days after treatment (Fig. 1). From 5 to 12 days after treatment the aerial parts usually wilted, depending upon the moisture and temperature conditions in the greenhouse. In some cases the living part of the stem acted as a cutting and sent out roots near the soil line and just above the region of collapse. The reestablishment of an injured tomato in this manner often



FIGURE 1. Comparison of injuries on tomato due to residues of illuminating gas left in the soil and those caused by flowing gas and residues. (A) Seedlings placed in soil previously gassed. (B) Roots of seedlings in soil and subjected to flowing gas. Left to right in (A) and (B): control, one cubic foot, two cubic feet.

occurred after the stem had fallen over on the soil or on the edge of the pot.

The roots of the woody plants (Table I) were badly injured, but the aerial parts showed no ill effects from these treatments except in the case

TABLE I
EFFECT ON POTTED PLANTS OF PASSING ILLUMINATING GAS AND CERTAIN OF ITS
UNSATURATED HYDROCARBON CONSTITUENTS THROUGH THE SOIL FOR A
PERIOD OF 30 MINUTES

Plant	Vol. of gas in cu. ft.	Relative degree of injury to roots caused by different gases†						
		Illuminating gas			Ethyl- ene	Propyl- ene	Butyl- ene	Acety- lene
		Not filtered	Filtered through					
			Water	NaOH				
Tomato	1 4	+++ +++*	+++ +++*	+ ++	o ++	o ++	o ++	+ ++
Willow	1 4	++ +++	++ +++	- +	o +	+ +	+ +	++ +
Maple	1 4	++ +++	+ +++	- +	o ++	++ +++	++ +++	++ +++
Cherry	1 4	++ +++	- -	- ++	o +++	+ +	++ +++	++ +++
Silver bell	1 4	+ +++	- -	- +	o +	++ ++	+ ++	+ +++
Privet	1 4	+ +++	- ++	- +	o +	+ +	+ ++	+ +

† ° signifies no noticeable injury, + slight discoloration, ++ noticeable discoloration and death of part or all of many roots, +++ all roots badly discolored and most slender roots killed.

* All underground parts killed.

of willow and maple. Four cubic feet of illuminating gas caused the roots of willow to turn brown and killed all leaves and the upper part of the stem. Some of the middle-aged leaves of the maple became discolored at the tips and along the margins of the segments. All of the woody plants (Table I) subjected to four cubic feet of illuminating gas recovered in from three to four weeks as evidenced by a normal growth of existing shoots or of new shoots and the appearance of new roots in the soil. Ten cubic feet of illuminating gas killed all five species of woody plants. In contrast to these results the Boston fern was not killed by subjecting the roots in soil to ten cubic feet of illuminating gas. Only the oldest leaves were injured. Since the aerial parts of the fern were not protected against the vapors emanating from the soil during treatment, as was the case with the species of plants (Table I) having a central stem, it is not known how

much of the injury to old leaves was due to a toxic action in soil and how much to the escaping vapors which passed over the leaves. In a previous report (22) the fern was shown to be resistant when the leaves were confined with 4 per cent illuminating gas. Thus the Boston fern has proved to be highly resistant to illuminating gas in soil or when the aerial parts were confined with the gas.

Removal of toxic materials from illuminating gas. If the roots of potted plants were subjected to illuminating gas after the gas had first been passed through soil, moss peat, sodium hydroxide, or water, the injuries were less pronounced or failed to occur, depending particularly upon the total amount of gas and the rate of flow. Water was the least effective in reducing injury and sodium hydroxide was the most effective. Soil and moss peat were about equally effective. Japanese maples in eight-inch pots were killed by passing 50 cubic feet of illuminating gas through the soil in 10 to 11 days. By first passing the gas through ten liters of either soil or moss peat, the same amount of gas caused little or no injury and only slight injury occurred when 100 cubic feet of illuminating gas were first passed through soil or moss peat. Three liters of water did not prevent injury with 50 cubic feet of gas but the appearance of injury on aerial parts was delayed a few days. Scrubbing 100 cubic feet of the gas with water did not prevent the maple from being killed. When the amount of illuminating gas was just sufficient to cause slight or moderate injury, water was effective in reducing or eliminating this injury. Although water was effective in absorbing some of the toxic constituents when the gas was flowed through at the rate of one to two cubic feet per day (Fig. 2), it was not effective when a rapid flow of gas was used (8 cubic feet per hour) as shown in Table I.

Since a more specific type of injury was produced by flowing the illuminating gas over the aerial parts of tomato than resulted from an exposure of the roots in soil to the flowing gas, the former method was used to determine the critical limits for removal of the highly toxic substances, particularly HCN, from the artificial gas. Flowing one cubic foot of illuminating gas in 12 minutes over the aerial parts of a young tomato plant under a bell jar caused permanent downward curling and coiling of the leaflets on upper leaves. When one cubic foot of gas was passed over the aerial parts in 30 minutes or at a slower rate, most of the leaves were killed. In control tests with nitrogen gas one cubic foot caused no injury. One-fourth cubic foot of illuminating gas was approximately the minimum amount which caused slight discoloration of the veins on the lower portion of young tomato leaflets during a period of exposure of 20 to 30 minutes. Complete protection against injury from one cubic foot of illuminating gas was obtained by first passing the gas through alkali hydroxide or silver nitrate and then through water. Water alone was not effective in

removing the highly toxic substances from the same amount of the gas. One cubic foot of nitrogen gas first passed through alkali hydroxide or silver nitrate and then through water caused no injury to the aerial parts of the tomato.

The effectiveness of alkali hydroxide or silver nitrate as scrubbers in removing highly toxic substances from illuminating gas was limited with respect to the total amount of gas, the rate of flow, the period of exposure, and the number of cylinders in series containing the scrubber solutions.



FIGURE 2. Effectiveness of different media in absorbing the toxic constituents from 11 cubic feet of illuminating gas previous to its passage through soil in eight-inch pots containing large tomato plants. The media used and the time of exposure to gas are from left to right: control, 13 days; 1 liter 15 per cent sodium hydroxide, 13 days; 6 liters moss peat, 6 days; 6 liters soil, 9 days; 1 liter of water, 12 days; and no medium used (roots and base of stem killed).

In order that lack of oxygen should not become a limiting factor, air was admitted simultaneously with the illuminating gas and at approximately the same rate. Drying effects on the tomato leaves were prevented by first passing the gas and the air through water just previous to their entrance to the bell jar. Under conditions in which the scrubbed illuminating gas injured tomato leaves, scrubbed nitrogen gas caused no injury. Since alkali hydroxide and silver nitrate were used primarily to remove hydrocyanic acid from the illuminating gas, tests for this constituent were made on the contents of each of the four cylinders in the scrubber system. Al-

though the potassium hydroxide in the first cylinder removed most of the hydrocyanic acid, small amounts of this gas passed into the second cylinder but not into the third and fourth cylinders, provided the rate of flow was not more than one and one-half cubic feet per hour and the total amount of illuminating gas was not in excess of ten cubic feet. Under similar conditions a positive test for cyanide was never obtained beyond the first cylinder of silver nitrate. A positive test for cyanide was not obtained when a strip of copper-benzidine acetate paper was exposed to the scrubbed illuminating gas in the bell jar although the unscrubbed gas gave a good test. The aeration of an alkaline solution of 1 per cent KCN showed that no cyanide was removed in the form of HCN. Even though complete removal of hydrocyanic acid was indicated by these tests, amounts of the scrubbed illuminating gas in excess of five cubic feet produced injuries to the tomato leaves that were the same as those caused by the unscrubbed gas. Five cubic feet was approximately the largest amount of illuminating gas which could be rendered non-toxic by the scrubbers when the gas was passed through at the rate of three-fourths to one and one-half cubic feet per hour.

Unsaturated hydrocarbons. When ethylene, acetylene, propylene, and butylene were passed through soil in amounts of less than one cubic foot, no injuries occurred, consequently larger amounts were used. A comparison of the effects of illuminating gas with those produced by each of four unsaturated hydrocarbon gases is given in Table I for plants subjected to one and to four cubic feet of flowing gas passed through the soil in 30 minutes.

Ethylene and propylene were the least toxic of the four unsaturated hydrocarbons listed in Table I. It has been estimated that ethylene constitutes approximately three per cent of the illuminating gas used in these experiments (10, p. 156). On this basis one cubic foot of pure ethylene would represent 33 times its concentration in one cubic foot of illuminating gas. Four cubic feet of ethylene caused injuries less severe in most cases than resulted from one cubic foot of illuminating gas (Table I), yet this amount of ethylene represents 133 times its concentration in one cubic foot of the artificial gas. Ten cubic feet of ethylene was not injurious to tomato seedlings (Fig. 3).

Each of the other three unsaturated hydrocarbons is usually present in illuminating gas in amounts of less than 1 per cent, acetylene being approximately 0.05 per cent (1, p. 133). Although the data in the last three columns of Table I show that these particular unsaturated hydrocarbons in many cases produced injuries comparable to those caused by an equal volume of illuminating gas, the amounts used represent several hundred times the concentration of each in illuminating gas.

Carbon monoxide. The maximum amount of carbon monoxide used was

two cubic feet. This amount was not injurious to young tomato seedlings grown in four-inch pots.

Nitrogen. When four cubic feet of nitrogen were passed through soil in 30 minutes the injury to roots of plants listed in Table I was so slight as to be questionable in most cases.



FIGURE 3. Effect on the tomato of residues left in soil by ethylene and by illuminating gas. Left, control. Middle, 10 cubic feet of ethylene. Right, collapse of roots and lower part of stem in soil caused by the residues from three cubic feet of illuminating gas. Photographed six days after seedlings were planted in the soil.

TOXICITY OF RESIDUES FROM ILLUMINATING GAS

Residues left in water. When from one to five cubic feet of illuminating gas were passed through one liter of tap water, residues remained which killed the roots and the stems of tomato cuttings or seedlings that were placed in 180 cc. of this solution after the flow of gas was stopped. Ethylene, propylene, acetylene, natural gas (mostly methane), and carbon monoxide tested in a similar manner did not kill the roots of tomato cuttings. Water charged with any one of these gases retarded root growth, caused various types of bending and coiling of roots, stimulation of branch roots, and the appearance of many abnormally long root hairs near the

root tip. The highly toxic effects of water charged with illuminating gas were eliminated by first scrubbing the gas with potassium hydroxide.

When not sufficiently toxic to kill the roots, water charged with illuminating gas caused these responses also. The highly toxic constituents disappeared rapidly from gassed water left in the open, but root bending was induced by ethylene-water that had remained in an open pan for 48 hours. A presentation time of one minute to water charged with ethylene was sufficient to cause bending of the roots. In this test only a portion of the root was lowered into the water containing dissolved ethylene and after one minute the roots were removed, washed in running tap water for 40 seconds, and then placed in fresh tap water. From three to eight hours were required for the bending response of the roots.

Residues left in soil. Injuries to plants transferred from control soil to gassed soil were essentially the same as those resulting from an exposure of the roots in soil to flowing illuminating gas, but the degree of injury was greater in the latter case provided the plant was not removed from the treated soil. Wilting, drying, and yellowing of leaves and retardation of growth of the aerial parts were the main symptoms visible on above-ground parts of plants placed in gassed soil. Injurious effects of any sort involved injury to the roots, but there was frequently severe injury to roots without any visible symptoms appearing on the aerial parts (Fig. 1). Epinasty, leaf-fall, and proliferation were usually not pronounced on these plants. In the case of tomato many roots appeared on the stem at and above the soil line.

When tomato seedlings were planted in pots of different size, the gassed soil in large pots produced more injury than the same soil in small pots. If, however, the same amount of gas (for example, four cubic feet) was passed through different volumes of soil, the smallest volume was the most toxic. There appears to be no arithmetical relation between the volume of soil gassed and the amount of gas used. For example, four cubic feet of gas passed through 500 g. of soil produced collapse of tomato seedlings, but eight cubic feet passed through 1000 g. of soil did not cause collapse of the plants. Approximately 15 cubic feet of gas were required to produce collapse of the tomato in 1000 g. of soil. Gassed soil which produced no marked injury on roots frequently stimulated the growth of tomato plants.

The type and degree of injury varied with the species and with the age of the plant. Herbaceous plants were more severely injured than woody plants when they were placed in the same lot of gassed soil. Young actively growing plants showed greater injury than older or slower growing plants of the same species. For a given plant the toxicity of gassed soil depended upon the total amount of illuminating gas used, the rate of flow, the volume of soil gassed, and the volume of gassed soil in which the plant was placed.

The passage of illuminating gas through soil in three two-liter flasks arranged in series rendered the soil in the first flask more toxic than the soil in the second or third flask. There was relatively little difference in toxicity of the soil in the second and third flasks as compared to that for the soil in the first and second flasks. Toxicity of this soil was determined by its effect on seedling tomatoes. In a similar manner the soil in any closed container was found to be more toxic nearest the inlet of illuminating gas as compared with the soil nearest the outlet.



FIGURE 4. Comparison of seed germination and seedling tests for determining the relative toxicity of soil through which illuminating gas had been previously passed. (A) Buckwheat and tomato grown from seed planted in soil through which 0, 1, 5, and 15 cubic feet (from left to right) of illuminating gas had been passed. (B) Tomato seedlings planted the same time as seeds and in soil treated as described in (A).

Freshly-gassed soil containing residues from 1000 to 35,000 cubic feet of illuminating gas retarded or prevented the germination of seeds. Water extracts of this soil were also toxic to the seeds. The first 200 cc. of leachings were more toxic than subsequent portions. In gassed soil which had been leached with tap water the percentage of germination of tomato and buckwheat seeds was frequently higher than in gassed soil which was not leached. Leachings from the gassed soil usually retarded germination but

the final percentage of germination was often the same as for the controls. Soil subjected to smaller amounts of illuminating gas was less toxic to seeds and under certain conditions stimulating effects were observed. Gassed soil which was just sufficiently toxic to cause collapse of young tomato seedlings was not noticeably toxic to tomato and buckwheat seeds (Fig. 4).

A poor top soil which could not grow tomato plants to maturity caused the development of dark spots and streaks on or along the veins of tomato leaves. Gassing this soil before planting the seedlings caused the lesions

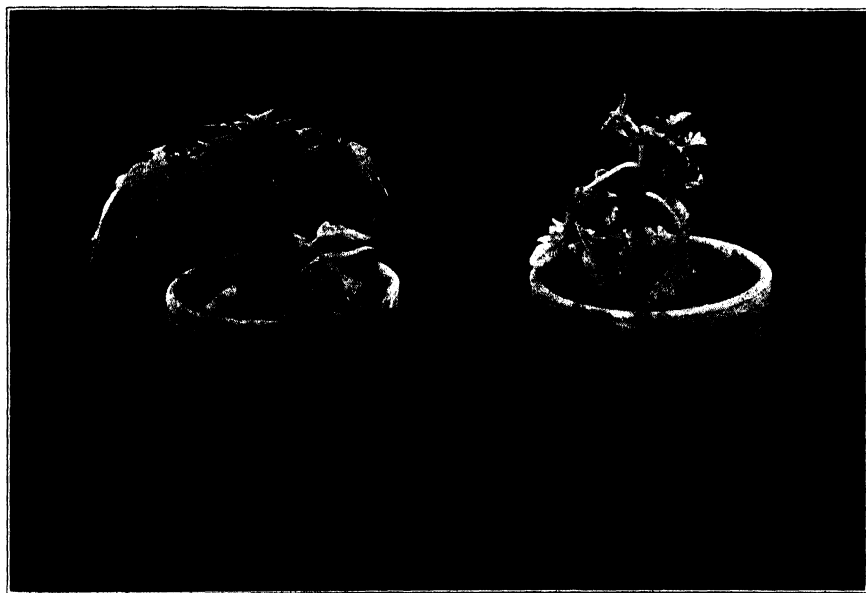


FIGURE 5. Injuries on the tomato caused by residues from illuminating gas remaining in the soil. Left, control. Right, seedling planted in gassed soil which had been stored in a sealed container in the laboratory for one week. This type of vein necrosis may be caused by poor soil but it appeared earlier when such soil had been gassed.

to appear from one to two weeks earlier and the symptoms were more pronounced (Fig. 5). Soil containing a normal amount of the common fertilizer elements did not cause this type of injury on the tomato regardless of whether the medium was gassed.

Vapors emanating from freshly-gassed soil injured the leaves of tomatoes when both were confined under a bell jar. The injury caused by vapors from 150 cc. of gassed soil after 24 hours is shown in Figure 6. The upper leaves were affected first and in those cases where only slight injury occurred, the lower part of the youngest leaflets was the only part to be injured. In general, mild injury involved a discoloration of the lower mid-

vein, main side veins, and adjoining tissues of the youngest leaflets; moderate injury was characterized by collapse of the petioles or of the lower portion of the mid-vein of the young leaflets and some of the middle-aged leaflets; and in cases of severe injury all upper leaves were killed and the lower leaves were either killed or badly injured.

Relative toxicity of illuminating gas and its residues. Under certain conditions the injuries due to toxic residues left in the soil were more pronounced than those caused by the flowing gas. In order to demonstrate this fact it was necessary to use an amount of gas and a period of exposure which caused a slight but definite type of injury when the roots in soil were exposed to flowing gas and the plant then transferred to control soil. To produce such an effect usually a larger amount of gas was required

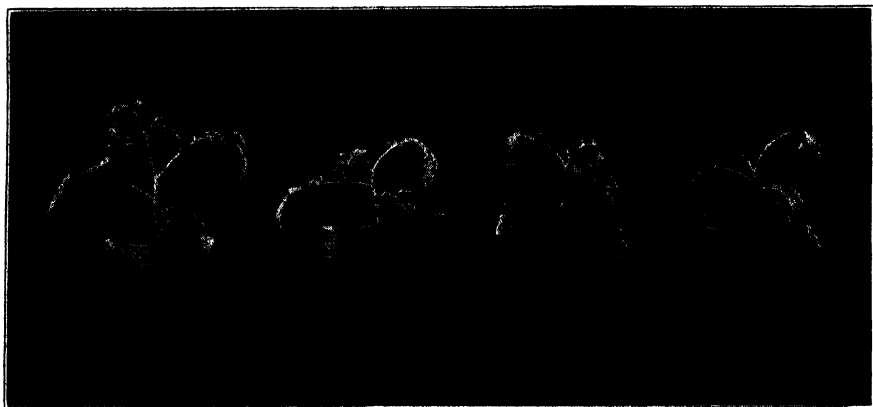


FIGURE 6. Injury on aerial parts of tomato caused by vapors from a 150-cc. sample of soil through which illuminating gas had been passed. The gassed soil and the plant were left under a bell jar for 24 hours. Left, control. The three plants on the right show increasing degrees of injury on the youngest tissues due to an increasing amount of gas which was passed through the soil samples.

for woody plants than for herbaceous types. Privet grown in four-inch pots was killed by flowing eight to ten cubic feet of illuminating gas through the soil in one hour. Another set of privet given the same treatment was exchanged, immediately after the flow of gas was stopped, with a set of plants growing in control soil. Both sets of plants received the same mechanical disturbance. The plants transferred from gassed soil to control soil showed injury to the roots but no injury to the aerial parts. The plants transferred from control soil to gassed soil showed more root injury, the older leaves turned yellowish and some of them fell from the plant, and the growth of shoots was retarded. After 14 days these two sets of plants had produced new roots but the plants in gassed soil continued to exhibit symptoms of injury in the form of a slight yellowing of

the older leaves and the failure to form new leaves. The response to the two treatments just described as contrasted with that caused by the combined treatments, in which case the plants were killed, shows that injuries to plants may be caused not only by flowing illuminating gas but also by the residual products remaining in the soil. Similar results were secured with young tomato seedlings and the persimmon. Results for the persimmon are shown in Figure 7. The combined treatment of flowing gas and toxic residues in (A) killed the plants within three days. Although



FIGURE 7. Comparative effects on the persimmon of subjecting the roots in soil (B) to 10 cubic feet of illuminating gas flowed through in 45 minutes; (C, D) to toxic residues from the same amount of gas; and (A) to the combined treatment of flowing gas and toxic residues. (A) Repotted in same soil. (B) Transferred after gassing to control soil. (C) Transferred from control to freshly-gassed soil. (D) Same as (C) but transfer made one day later.

abscission of the lower leaves occurred, there was no yellowing of the leaves such as resulted from the toxic residues as shown in (C) and evidenced by clearing of the veins on lower leaves. Plant (C) eventually lost all leaves. The roots of plant (B) were subjected to the same amount of flowing gas as in (A) but immediately after the flow of gas was stopped, plant (B) was transferred to control soil with the result that no injury was produced on the aerial parts.

Loss of toxicity from gassed soil. Gassed soil was rendered less toxic by storage in an open container at high temperatures (20° to 80° C.), by aeration with nitrogen gas, or by leaching with water. Soil just sufficiently toxic to cause collapse of tomato seedlings was either non-toxic or only slightly injurious to the seedling roots after it remained for one day in a pot in the greenhouse. A sealed sample from the same lot of soil lost most of its toxicity after being held one week in the laboratory, but the sealed samples stored for one week at 3° C. and at -15° C. were sufficiently toxic to cause collapse of the seedlings (Fig. 8). If after tomato seedlings were planted in gassed soil, escape of toxic vapors was retarded or prevented by pouring a layer of paraffin on the surface of the medium and by coating



FIGURE 8. Effect of low temperature storage on the toxicity of soil through which illuminating gas had been previously passed and which was then sealed for one week. Left to right: control soil -15° C., gassed soil -15° C., gassed soil 3° C., gassed soil 24° C. The tomato plants in gassed soil previously stored at -15° C. and at 3° C. were killed, but the soil kept in the laboratory (24° C.) caused only a slight retardation of growth.

the outside of the pot with the same material, greater injury resulted than was produced by the same soil not sealed in with paraffin or by control soil sealed with paraffin. Soil through which ten cubic feet of illuminating gas had been passed lost most of its toxicity after remaining in a pot in the greenhouse for 24 hours. The effect on the persimmon of the freshly-gassed soil and the same soil after storage for one day in the greenhouse is shown in Figure 7 C and D, respectively. Similar results were obtained with tomato and privet.

A 65-liter lot of soil, through which 35,000 cubic feet of gas had been passed, was sealed for a period of six months. Although at the end of this period the soil gave off distinct illuminating gas odors, it was not noticeably toxic to tomato seedlings. No immediate symptoms of injury de-

veloped but at the end of ten days the seedlings in the gassed soil were seen to be stunted as compared with those in control soil.

Soil through which 7000 to 35,000 cubic feet of illuminating gas had been passed was rendered non-toxic more readily by storage for three to five days in an open container at room temperature or placing in an oven held at 80° C. than by leaching with water. After leaching 500 g. of the gassed soil with 5000 cc. of water, the medium was still sufficiently toxic to cause the retarded growth shown on the right in Figure 9.



FIGURE 9. Effect on growth of tomato of leaching highly toxic gassed soil with water. Left to right: control; soil from a 65-liter lot through which 7210 cubic feet of illuminating gas had been flowed; a similar sample of gassed soil after leaching with an amount of water equal to ten times its volume. Photographed three weeks after planting.

Soil made sufficiently toxic by illuminating gas to cause collapse of tomato stems was aerated with nitrogen gas for the purpose of removing any toxic vapors which may have remained in the soil interstices. This procedure rendered gassed soil less toxic, but not non-toxic. The relative reduction in toxicity depended principally upon the amount of illuminating gas used and not the amount of nitrogen gas subsequently passed through the soil. When soil that was subjected to four cubic feet of illuminating gas was aerated with the same amount of nitrogen gas, collapse of tomato stems did not occur, but the plants showed other types of injury such as marked stunting and wilting or yellowing of the foliage. Collapse of tomato stems was not prevented by aeration of gassed soil with nitrogen

if the amount of illuminating gas was 50 cubic feet instead of four cubic feet. Approximately the same reduction in toxicity secured by aeration of gassed soil with nitrogen gas was accomplished by exposure of the gassed soil in a layer one-half inch thick to air for one hour.

Vapors from gassed soil causing the injury shown in Figure 6 and giving a positive test for cyanide were not toxic after 24 hours to a new set of tomato plants placed under the bell jars with the same lots of soil. At the end of the first 24-hour period, vapors from the soil failed to give a positive test for cyanide with copper-benzidine acetate paper.

Loss of toxicity from gassed soil was generally associated with the intensity of odors given off by the soil. Soil which gave off sharp odors of illuminating gas usually caused some injury to the roots of plants but the medium was not necessarily highly toxic. Loss of toxicity from the gassed soil was also associated with loss of hydrocyanic acid as shown by tests with the copper-benzidine acetate paper. That the injuries were not necessarily due to the substances responsible for the strong odors was shown by the fact that when the illuminating gas was first passed through sodium hydroxide, injuries to the plant were greatly lessened or prevented without affecting the intensity of the odors. Hydrocyanic acid disappeared more rapidly than the sharp illuminating gas odors from sealed samples of gassed soil.

Gassed soil which was injurious to tomato seedlings not only lost its toxicity in open storage, but frequently produced a stimulated growth. Tomato and buckwheat seeds germinated sooner and appeared above soil in a shorter time in gassed soil than those in the control soil. These results were repeated several times with 500 g. lots of soil through which four cubic feet of illuminating gas had been passed. Seedling tomatoes planted the first day were killed whereas those planted the second and third days in the same soil grew to be larger than the control plants.

ADDITION TO SOIL OF COMPOUNDS PRESENT IN ILLUMINATING GAS

Cyanides. In high concentrations calcium or potassium cyanide caused collapse of the underground parts of tomato in from 8 to 48 hours. Injuries produced by cyanide were essentially the same as those caused by soil through which illuminating gas had been passed. These soils lost their toxicity under the same conditions as those described for soil through which illuminating gas had been passed.

Four to seven milligrams of CN, added to the soil as $N/25$ KCN, was the minimum amount which caused collapse of young tomato stems. The corresponding minimum amount of illuminating gas (0.75 to 1.0 cubic foot) causing the same response contained from five to ten milligrams of CN. During the period of these particular tests the CN content of the illuminating gas varied from six to ten milligrams per cubic foot. The

amount of CN in the minimum toxic quantity of illuminating gas was usually in excess of that present in the minimum toxic dose of KCN, but the difference in comparable tests was usually not in excess of two times. Lesser amounts of CN were toxic but did not cause the collapse of tomato stems.

Benzol, toluol, xylol, and phenol. Benzol, toluol, and xylol produced injuries to roots and aerial parts similar to those caused by gassed soil or by calcium or potassium cyanide. Phenol produced similar injuries but in addition caused a pink or deep red coloration of the roots, particularly of privet, tomato, and sunflower. In high concentrations phenol in soil caused a deep pink or reddish discoloration of the roots, tap root, stem, and leaves of the tomato. In some cases the vascular system was discolored so that it could be readily seen from the outside and traced up the stem. This is the only treatment in which it was visibly evident that a known toxic material from the soil was transported to the leaves where it produced local injury and was the only one of the substances added to the soil which caused a pink coloration on roots similar to that caused by illuminating gas. Benzol disappeared most rapidly from the soil and phenol was the slowest to leave.

Drip oil. Drip oil obtained from a gas company and a product with similar characteristics obtained from our illuminating gas by low temperature fractionation produced injuries similar to those caused by gassed soil. Equal amounts of these two solutions were more toxic than toluol, xylol, or benzol. When added to 450 g. of soil (wet weight), all amounts of the drip oil in excess of one cubic centimeter caused collapse of young tomato seedlings during a period of 24 hours. Although small amounts of drip oil were highly toxic when added to soil, equivalent amounts of illuminating gas proved to be from 100 to 150 times as toxic. For example, one cubic foot of illuminating gas passed through 450 g. of soil caused the collapse of young tomato seedlings planted in this soil, yet one cubic centimeter of drip oil (equivalent to 147 cubic feet of illuminating gas) was the minimum quantity which was highly toxic to the seedlings. Amounts of drip oil in excess of the minimum toxic quantity rendered the soil toxic for relatively long periods. Soil to which seven cubic centimeters of drip oil had been added caused collapse of young tomato seedlings up to the twelfth day. Twenty-five cubic centimeters of drip oil rendered the soil (450 g.) highly toxic for 60 days.

Effect of vapors from the compounds added to soil. Minimum toxic concentrations of vapors from potassium cyanide, phenol, naphthalene, xylol, and drip oil injured the basal portion of the youngest leaflets located at or near the growing tip of tomato plants. Generally this injury was confined to the veins or adjacent regions. The discoloration caused by xylol and drip oil was usually lighter than that caused by the other three vapors.

Frequently naphthalene produced a permanent curling of the young leaflets similar to that caused by flowing illuminating gas. This type of curling was caused by a localized injury to the under side of the mid-vein or main side veins. Higher concentrations of these vapors injured all leaves so badly that the specific symptoms characteristic for minimum doses were masked. There was an extremely narrow margin between the amount of the solution necessary to cause minimum injury and that which produced injury on all leaves. Equal amounts of xylol were more toxic than toluol or benzol.

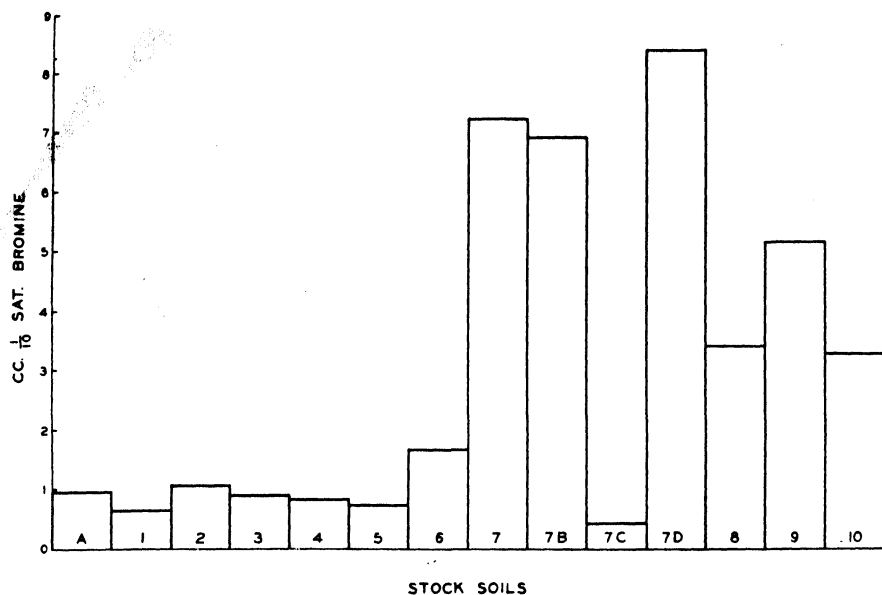


FIGURE 10. Variation in bromine absorption values for the extracts of 14 different control soils. Soils numbers 7, 7 B, and 7 D were sterilized and numbers 8, 9, and 10 were relatively high in organic matter. The remaining soils were low in organic matter and in fertilizer elements.

CHEMICAL METHODS FOR DETECTING ILLUMINATING GAS IN SOIL.

Heavy hydrocarbons. Extracts of gassed soil reacted with more of a one-tenth saturated solution of bromine than the extracts from similar soil which had not been gassed. The presence of a distinct color in most of the soil extracts, ranging from straw to dark brown, made it difficult to obtain exact values with the direct bromination method. Consequently, an excess of bromine was added to soil extracts which were colored and the excess titrated with sodium thiosulphate in the presence of potassium iodide, using starch as an indicator. When 14 different control soils were tested by this modified method, it was found that extracts of some of them

gave higher bromine absorption values than those of certain soils through which gas had been passed. The bromine values for extracts of these soils are shown in Figure 10. Extracts of soil which had been dried for several weeks either at room temperature or at 80°C. gave higher bromine values than extracts of the same soil maintained in a moist condition. This was true for both control and gassed soil samples. Extracts of control soils high in organic matter (numbers 8, 9, and 10 in Figure 10) and those of soil which had been recently sterilized (numbers 7, 7 B, and 7 D in Figure 10) gave high bromine absorption values. Soil 7 C is before and 7 D after sterilization.

Phenols. Extracts of gassed soil gave positive tests for phenols and those from certain control soils gave negative tests. When the extracts were of a deeper coloration than light straw, the results were unreliable since the color produced by diazobenzene sulphonic acid in the presence of small amounts of phenol was the same as the color of the soil extract.

Hydrocyanic acid and cyanogen. The illuminating gas used in these experiments contained from 9 to 21 grains of hydrocyanic acid per 100 cubic feet of gas. The average value was 16 grains per 100 cubic feet of gas. Qualitative tests for the presence of HCN in illuminating gas made at many different times during the last three years were always positive. The cyanide was in the form of hydrocyanic acid (HCN) and not as cyanogen (C_2N_2), since no test was obtained for cyanogen in 2 N potassium hydroxide in series with and after a 10 per cent solution of acidified silver nitrate. Similar tests on gassed soil were positive immediately after treatment but were negative a few days later, depending upon the amount of gas passed through a given volume of soil, and the conditions of storage. A soil which caused the copper-benzidine acetate paper to turn blue in a few seconds was toxic to plants placed in it. Soil which gave a negative test with this paper was either non-toxic or only slightly injurious as shown by a temporary retardation of growth of plants placed in it. Extracts of freshly-gassed soil usually did not show a positive test for cyanide if less than 50 cubic feet of gas had been used.

DISCUSSION

Illuminating gas injury through the soil has been attributed to displacement of oxygen from the soil, to the unsaturated hydrocarbon gases (particularly ethylene) held in the soil interstices, and to the hydrocyanic acid in the gas. No one of these explanations has been universally accepted as accounting for all types of injuries resulting from the passage of illuminating gas through soil. As early as 1874 Boehm (2) referred to illuminating gas injury as being due to a poisoning of the soil. Since that time it has not been established whether the plant is injured or killed mainly by the residues left in soil or by the flowing gas.

Results with short periods of exposure to a rapid flow of nitrogen gas showed that before lack of oxygen became a limiting factor, the toxic substances in illuminating gas had caused severe injury to the root system. When illuminating gas escapes from leaks in gas mains, lack of oxygen would probably be of less importance since the physical restrictions occurring in the field are not likely to be so effective as the walls of a pot in confining the gas to the vicinity of the roots of a plant. Under field conditions, therefore, complete displacement of oxygen from the soil would probably not occur. In order to show that lack of oxygen in gassed soil was the principal cause of toxic effects on plants, it would be necessary to demonstrate by actual measurement that the oxygen content of the gassed soil was below the minimum oxygen requirement of the roots.

Some of the responses, particularly epinasty, leaf-fall, and yellowing of leaves are known to be produced by an exposure of the plant for several hours or longer to carbon monoxide (23) and to the unsaturated hydrocarbon constituents of illuminating gas such as ethylene, propylene, and acetylene (4). But no one of these constituents caused injury when used in an amount equivalent to its occurrence in illuminating gas when the period of exposure was one hour or less. The elimination of these particular constituents as a possible cause of the principal injury was dependent upon the use of short periods of exposure, since a period of exposure of several hours or longer caused ethylene responses which masked or made more difficult the determination of responses due to other causes.

Toxicity of gassed soil was due primarily to constituents of illuminating gas which combined with the soil particles during the period of gas flow and not to vapors which remained in the soil interstices as proposed by Harvey and Rose (8). After combining with the soil these toxic constituents were not readily removed by aeration or by leaching with water, but eventually the gassed soil became non-toxic even when stored in a sealed container. These results indicate that the original form of the toxins in gassed soil changed to less toxic forms. Since the displacement of vapors in the soil interstices reduced the toxic effects of gassed soil, aeration has been recommended as the initial procedure in rendering gassed soil less toxic.

Since sealed samples of gassed soil held at room temperature lost their toxicity more rapidly at room temperature than at 3°C . or at -15°C ., it would appear that the change in toxicity might be of a biological nature. The hydrocyanic acid or cyanide may have been changed to other forms of nitrogen by soil organisms. Other toxic constituents might also be changed by the action of soil organisms.

Injuries produced by the addition of calcium or potassium cyanide to soil were similar to those caused by illuminating gas. Since phenol, xylol, toluol, benzol, and drip oil also caused similar injuries, the response to cyanide in soil was not a specific one.

Scrubbing the illuminating gas with either alkali hydroxide or silver nitrate was primarily for the purpose of removing hydrocyanic acid. The collapse of seedling tomato stems in soil or of roots and stems of tomato cuttings in water did not occur when the illuminating gas was scrubbed with alkali hydroxide. Although HCN was removed from ten cubic feet of illuminating gas, this amount of the scrubbed gas produced the same type of injury on the actively growing leaflets of the two upper leaves of the tomato as was caused by the unscrubbed gas. Since one to five cubic feet of illuminating gas could be rendered non-toxic by the scrubbers and six to ten cubic feet could not, it would appear that some constituent other than HCN causes a similar type of injury on tomato leaves, or that traces of HCN, less in amount than are required to give a positive Prussian blue or thiocyanate test, passed through the scrubbers.

It was shown that one-fourth of a cubic foot of unscrubbed illuminating gas was approximately the minimum amount which produced a slight but specific type of injury to tomato leaves. The corresponding minimum amount of the scrubbed gas is between five and six cubic feet. On this basis the unknown toxic constituent of illuminating gas which passed through the scrubber is from 20 to 24 times less effective but produces the same type of injury as the HCN in the unscrubbed gas.

Tests in which known amounts of KCN were added to the soil showed that the minimum quantity of CN causing collapse of tomato stems was approximately the same as that which was present in the minimum amount of illuminating gas causing the same response. It is believed, therefore, that the highly toxic action in soil of the illuminating gas used in these experiments was due mainly to cyanide. The toxicity of artificial illuminating gases from which all cyanide had been removed would probably be due mainly to the unsaturated hydrocarbons, principally ethylene, and to carbon monoxide. If large amounts of a cyanide-free artificial illuminating gas escaped into soil over a long period of time, in addition to injuries of the ethylene-induced type there would no doubt be toxic effects caused by other constituents, even by such relatively inert substances as the saturated hydrocarbons. Constituents of the gas which have no direct toxic effect on the roots may play a part indirectly by changing the solubilities of ions or otherwise altering the chemical relationships in the soil. It is possible also that the biological processes might be affected. The slightly stimulating effects on the tomato of gassed soil which had become non-toxic upon standing for a few days, may be due to either or both chemical or biological changes caused by the gas.

Schollenberger (14) found that a natural gas composed mainly of methane and ethane rendered soil highly toxic to wheat and oats. Analysis of this soil showed a marked increase in soluble manganese and ammonium nitrogen, and lesser but distinct increases in sodium, potassium, and cal-

cium. Since these changes frequently occur as a result of water-logging, puddling, or other conditions which favor reducing actions, Schollenberger concluded that the toxic effect of the natural gas in soil was due primarily to a reduced oxygen pressure. No data concerning the oxygen content of the soil were given. After remaining for six weeks in a closed (not strictly air-tight) container, the gassed soil lost not only its toxicity but proved to be slightly stimulating to wheat. Analysis of the soil at this time showed that soluble manganese had practically disappeared. The question arises as to whether the increase in soluble materials, particularly manganese, may not have been the chief cause of the toxicity and not lack of oxygen. Conclusions similar to those given by Schollenberger for the toxic action of natural gas were arrived at earlier by Ehrenburg and Schultze (7, p. 81) for the toxic effects of artificial illuminating gas in soil.

The many more difficulties involved in the diagnosis of illuminating gas injury through the soil as compared to the diagnosis of injury caused by contact of aerial parts of plants with an atmosphere containing the gas are due to distinct differences in the mode of action of the gas on plants. In the latter case the principal constituent (ethylene) causing injury is known and the detection of this gas in low concentrations which are toxic to aerial parts is readily accomplished by the tomato test (3). When injury results from the passage of illuminating gas through soil, hydrocyanic acid and not ethylene becomes of major importance but at present all of the toxic constituents are not known with certainty and no simple test has been devised which can show that toxic concentrations of illuminating gas existed in the soil long enough to produce injury to the plant.

While tests with tomato plants may be useful in detecting the presence of ethylene in soil or escaping from soil, this method would not be a measure of the relative toxicity of the soil. Experimental results with small volumes of soil show that relatively large amounts (several cubic feet) of illuminating gas must be passed through soil in order to produce an injury equivalent to that caused by confining the aerial parts with traces (less than one to a few cc.) of the gas. The tomato plant test recommended by Crocker (3) is designed primarily for detecting traces of ethylene and will show whether or not the air contains enough of the gas to cause injury, particularly epinasty and abscission of leaves, to aerial parts of plants without reference to root injury. In the case of illuminating gas injury through the soil, root injury is of primary importance.

Field diagnosis should not only establish the presence of the gas in soil, but it must be shown that the amount of gas is sufficient to cause a given type of injury. In general the responses of plants to the passage of illuminating gas through soil were non-specific, being in many cases identical with those due to lack of moisture, high temperature, or both. Specific responses such as epinasty and abscission of at least some green leaves oc-

curred only with a slow flow of gas (2 to 5 cubic feet per day). Higher rates of flow killed the leaves and usually some branches but caused little or no epinasty and abscission. Since with a rapid rate of flow (in excess of 25 cubic feet per day) the toxic action causing wilting and drying of leaves operated more rapidly than the processes involved in producing epinasty and abscission, the principal injuries on aerial parts were not of the ethylene-induced type.

The heavy hydrocarbon and phenol soil tests designed to show that illuminating gas has been passed through the soil are of value in laboratory work, but they are decidedly limited in their application to field tests. Even though it can be established that illuminating gas has passed through the soil, it would not be a simple matter in all cases to show that the injury to certain plants was caused by the gas and not by some other agent.

So long as large amounts of illuminating gas are passing through soil, the medium would be toxic to new plants placed therein. However, after the flow of gas was stopped, the soil might be rendered suitable for replanting by digging or spading up to a depth of from one to three feet and allowing to remain in a fallowed condition for from one to ten days depending upon the relative amount of illuminating gas which had been passed through, the rate of flow, and the mechanical texture of the soil. This method would allow most of the entrapped toxic gases to escape. After sharp odors ceased to be given off, the soil could then be further reduced in toxicity by application of large amounts of water. The use of water alone, without being preceded by aeration of the soil, would require a longer time and would be of doubtful value except possibly in the case of noticeably porous soil types. In laboratory experiments an amount of water equal to at least ten times the volume of soil was required to render a highly toxic medium non-toxic as compared to a similar reduction obtained by allowing the soil to remain exposed to air (without watering) for three to five days.

Since tomato seedlings from two to four inches tall are much more sensitive to gassed soil than seedlings of most woody plants, the relative toxicity of gassed soil in the field could be determined by planting tomato seedlings in a four to six-inch pot of the gassed soil. Soil which was not noticeably toxic to these tomato seedlings would be suitable for replanting to trees or shrubs. In such a test the sample of gassed soil should be taken at least six inches under the surface and then placed at once in a sealed container and carried to the greenhouse where planting of the tomato seedling should be done immediately. Seed germination tests were found unreliable in determining the relative toxicity of gassed soil.

SUMMARY

1. When plants were injured by illuminating gas in soil, as compared to confinement of the aerial parts with the gas, the conditions prevailing

were distinctly different with respect to the minimum toxic amount of gas, the principal injurious constituents, parts of the plant first affected, type and degree of injury, and the minimum period of exposure.

2. Initial injuries resulting from a slow flow (1 to 5 cubic feet per day) of illuminating gas consisted mainly of ethylene responses such as epinasty, yellowing, and the abscission of some green leaves. With higher rates of flow the initial response consisted of wilting, drying, or browning of leaves and there was little or no epinasty and no abscission of normal-appearing leaves. Roots of these plants were badly injured or killed.

3. Injuries were caused by the residual products of illuminating gas remaining in the soil as well as by the flowing gas. During periods of exposure of one hour or less to a rapid flow of the gas, more injury was caused by the residues in soil than by the flowing gas. The initial injury occurred on the roots regardless of whether the aerial parts were affected.

4. The highly toxic effects of gassed soil were not due to such constituents as ethylene, propylene, acetylene, butylene, carbon monoxide, or to lack of oxygen. In concentrations equivalent to their occurrence in a minimum toxic amount of illuminating gas, these constituents were not injurious. A period of one hour or less was not sufficient to induce ethylene responses on the aerial parts, but the roots were badly injured and in some cases also the tops by constituents other than the unsaturated hydrocarbon gases. Control tests with nitrogen gas showed that lack of oxygen was not a limiting factor during short periods of exposure to illuminating gas.

5. After passing illuminating gas through water, residues remained which killed the roots and lower part of the stem of tomato cuttings. Water charged with equal amounts of carbon monoxide, ethylene, propylene, acetylene, butylene, or natural gas (mostly methane) retarded root growth but did not kill the roots. In the dissolved state all of these gases and also sub-lethal amounts of illuminating gas produced various types of bending, coiling, and swellings on roots, stimulated the growth of branch roots, and caused the formation of a large number of abnormally long root hairs near the root tip. Water charged with illuminating gas did not kill tomato roots if the gas was scrubbed with alkali hydroxide.

6. Loss of toxicity from gassed soil was rapid when the medium was stored in the open at temperatures of 20° to 80° C. Sealed samples of gassed soil eventually lost their toxicity, but the loss was more rapid at room temperature (20° to 30° C.) than at 3° C. or at -15° C. Storage of gassed soil in the open for two to five days removed most of the highly toxic substances. An equivalent reduction in toxicity by leaching required a volume of water approximately ten times the volume of soil. Aeration of gassed soil with nitrogen gas rendered the soil less toxic but not non-toxic. Loss of toxicity from gassed soil was more closely associated with the disappear-

ance of hydrocyanic acid than with the intensity of odors emanating from the medium.

7. Removal of hydrocyanic acid by scrubbing the illuminating gas with alkali hydroxide or silver nitrate rendered small amounts (1 to 5 cubic feet) of the gas non-toxic to tomato leaves. Larger amounts of the scrubbed gas (6 to 10 cubic feet) produced injuries similar to those caused by the unscrubbed gas, even though complete removal of HCN was shown. An amount of scrubbed gas from 20 to 24 times the amount of unscrubbed gas was required to produce the same type and degree of injury.

8. The addition to soil of alkali cyanide, phenol, benzol, toluol, or drip oil produced injuries to the tomato similar to those caused by the passage of illuminating gas through soil. In 400 grams of moist soil the minimum amount of KCN which produced collapse of young tomato seedlings contained approximately the same quantity of CN as was found in the minimum amount of illuminating gas causing a similar response. This difference in the amount of CN was usually not greater than two times. Phenol was the only compound which produced a pink coloration of roots similar to that caused by illuminating gas.

9. Tests for the presence of heavy hydrocarbons in extracts of gassed soil and control soils showed that the degree of bromination was not a true measure of the heavy hydrocarbons. Control soils high in organic matter yielded brownish extracts which were not suitable for direct bromination tests and these extracts gave high values with a modified bromine absorption method. Tests for phenols in extracts of gassed soil were unreliable due to the presence of a color which was nearly the same as that of the dye indicator.

10. Some of the difficulties which may be encountered in diagnosis of illuminating gas injury through the soil in the field are discussed from the standpoint of the results obtained in laboratory experiments with potted plants.

11. A simple method is suggested for determining the relative toxicity of gassed soil, particularly with reference to replanting after the flow of illuminating gas has been stopped, which consists of planting young tomato seedlings in samples of the gassed soil.

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HASTENING THE GERMINATION OF DORMANT GLADIOLUS CORMELS WITH VAPORS OF ETHYLENE CHLORHYDRIN¹

F. E. DENNY AND LAWRENCE P. MILLER

In a previous paper (1) the results of preliminary tests on the effect of vapors of ethylene chlorhydrin upon freshly-harvested *Gladiolus* cormels (bulblets) were given. At that time successful treatment of the cormels had been obtained only after the removal of the hard outer coat. Subsequent experiments showed that good forcing could be obtained even with cormels having intact coats provided that suitable concentrations of chemical and periods of exposure were used.

But when the tests were made at irregular intervals upon such cormels as were available at the time, the results were not uniform. Although the treatment in each test hastened sprouting and increased the percentage of germination, the number of days of gain and the total germination varied widely in different tests. Until the causes of these variable results could be learned a definite procedure for the use of ethylene chlorhydrin with the cormels could not be recommended.

It seemed necessary to carry out systematic tests, beginning at once after the harvest of the cormels and continuing the tests at intervals thereafter until the cormels were no longer dormant, in order to determine the importance of the length of the natural after-ripening period upon the response of the cormels to chemical treatment. Furthermore, in the preliminary tests the sorting into sizes had been done only approximately, and it appeared that more rigid standards as to sizing of cormels might assist in explaining the variation in the sprouting response. The storage condition from the time of harvest until the treatments were applied was also considered as a possible factor influencing the response of the cormels.

Such an experiment required generous supplies of cormels of various sizes and of different varieties. These became available in October 1932. The present paper gives the results of such a test, and shows the conditions under which dormant gladiolus cormels can be treated in order to hasten germination, and indicates approximately the results that may be expected from such treatments with different varieties.

METHODS

Cormel size. As soon as they were harvested the cormels were sorted by screening into four sizes. The size designated in this paper as "large" passed through a screen of 0.420 inch mesh with wire whose diameter was 0.080 inch, but not through a screen 0.301 mesh with 0.032 wire; 100 of

¹ Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 71.

these cormels weighed 40 to 50 grams in the freshly-harvested condition. The size called "medium" passed through the screens mentioned but not through a screen with 0.187 mesh with 0.065 wire; 100 of these weighed 20 to 25 grams. The size called "small" passed through the screens mentioned but not through a U. S. No. 4 sieve with 0.187 mesh and 0.050 wire; 100 of these weighed 10 to 15 grams. The size called "very small" passed through all of the screens and 100 cormels weighed 6 to 9 grams. Only a few experiments were carried out with the very small size.

Storage. As soon as they were screened into sizes the cormels were counted out into lots of 100 cormels each in the case of the large size and 150 in the case of the small and medium sizes. These lots were placed in cloth bags and equal numbers of bags of each size were stored under two different conditions (a) in moist peat moss to prevent loss of moisture, (b) in shallow trays to facilitate drying-out of the cormels.

Treatments. At once after harvest and at intervals of three to four weeks thereafter bags of cormels were removed and placed in Mason fruit jars. In the space above the bags of cormels were placed pieces of cheesecloth into which measured amounts of 40 per cent ethylene chlorhydrin ($\text{ClCH}_2\text{CH}_2\text{OH}$) were placed. The amounts of chemical varied usually from 3 to 7 cc. per liter of volume of the jar and the period of treatment varied usually from three to seven days. The jars were sealed and at the end of the period of treatment the cormels were removed and planted in moist soil in flats.

Germination record. When sprouts began to appear the flats were placed in the greenhouse and at intervals of four to seven days counts were made of the number of sprouts that had emerged. From this record a graph was prepared for each lot showing the percentage germination at intervals after treatment. From these graphs were obtained the data shown in the tables in this paper.

RESULTS

LARGE, MEDIUM AND SMALL CORMELS

Per Cent Germination at Intervals

The experiments included treatments with three concentrations of 40 per cent ethylene chlorhydrin, 7 cc. per liter for seven days, 5 cc. for five days, and 3 cc. for three days. Although it was found that treatments with 7 cc. per liter for seven days were too strong, at least for all except Alice Tiplady cormels, successful forcing was obtained with 5 cc. for five days and 3 cc. for three days, these two concentrations being about equally effective. Each of these treatments was applied to cormels that had been stored in moist moss and to those that had been stored in dry condition. There was little difference between the results from moist and dry storage, although somewhat more rotting of cormels occurred in the moist than in

the dry condition. In order to make the data more easily understood, and amenable to presentation in tabular form without complexity, the tables were prepared from the data obtained from the treatments of dry-stored cormels by the use of 3 cc. per liter for three days.

Remembrance. The results with cormels of the variety Remembrance are shown in Table I. The treatments were effective at all periods of treat-

TABLE I
EFFECT OF ETHYLENE CHLORHYDRIN UPON THE GERMINATION OF
CORMELS OF VARIETY REMEMBRANCE

Size of cormel	Date of treatment	Days after treatment, per cent germination							
		60 days		100 days		140 days		180 days	
		Tr.	Ck.	Tr.	Ck.	Tr.	Ck.	Tr.	Ck.
Large	Oct. 17	0	0	76	13	76	48	76	60
	Nov. 7	38	0	82	6	82	12	82	14
	Nov. 30	22	0	87	0	87	0	87	0
	Dec. 20	78	0	96	0	96	0	96	6
	Jan. 16	49	0	82	3	82	40	82	63
Medium	Oct. 17	0	0	41	0	74	6	74	7
	Nov. 7	12	0	79	0	83	0	83	0
	Nov. 30	76	0	92	0	92	0	92	6
	Dec. 20	64	0	86	0	86	0	86	1
	Jan. 16	17	0	80	4	84	8	85	38
	Feb. 14	26	0	80	0	84	18	88	42
Small	Oct. 17	0	0	41	0	63	2	68	4
	Nov. 30	37	0	74	0	78	0	78	6
	Jan. 16	10	0	82	0	85	6	85	7
	Feb. 14	30	0	52	0	60	0	68	4

ment from October 17 to February 14 although germination began at a less early period for the first treatment after harvest, i.e., on October 17. In all cases the treatment showed good germination at 100 days after treatment at which time either none or only a few of the untreated cormels had appeared above ground. The results for the check lots, especially the large size (columns 7 to 10 at top), showed that the cormels when freshly-harvested were not entirely dormant and that they became more dormant during storage after harvest. Plantings on November 30 showed no germination even after 180 days. Subsequently the cormels became progressively less dormant until at the planting of January 16 fair germination was obtained in 140 days. The small size gave low germinations of untreated cormels even for the February 14 planting. But with this size the ethylene chlorhydrin treatments were successful not only on February 14 but even on October 17.

Souvenir. The results with the variety Souvenir are shown in Table II. The cormels of this variety were much less dormant than those of Re-

membrane. Thus, the untreated cormels showed good germination after about 100 days if planted after December and in about 180 days when

TABLE II
EFFECT OF ETHYLENE CHLORHYDRIN UPON THE GERMINATION OF
CORMELS OF VARIETY SOUVENIR

Size of cormel	Date of treatment	Days after treatment, per cent germination							
		60 days		100 days		140 days		180 days	
		Tr.	Ck.	Tr.	Ck.	Tr.	Ck.	Tr.	Ck.
Large	Oct. 17	0	0	15	0	83	30	92	77
	Nov. 7	0	0	95	2	98	48	99	76
	Nov. 30	40	0	90	12	90	67	95	79
	Dec. 20	62	0	80	38	80	66	80	79
	Jan. 16	34	22	84	90	84	92	84	92
	Feb. 14	54	54	82	82	82	82	82	82
Medium	Oct. 17	0	0	0	0	41	0	83	81
	Nov. 7	0	0	71	0	91	17	91	62
	Nov. 30	8	0	92	0	98	54	98	83
	Dec. 20	34	0	81	32	81	68	81	73
	Jan. 16	28	0	98	82	98	92	98	92
	Feb. 14	37	12	90	90	92	92	92	92
Small	Oct. 17	0	0	0	0	14	0	68	40
	Nov. 30	0	0	64	0	80	20	80	54
	Jan. 16	22	0	77	62	78	74	78	74

TABLE III
EFFECT OF ETHYLENE CHLORHYDRIN UPON THE GERMINATION OF
CORMELS OF VARIETY ALICE TIPLADY

Size of cormel	Date of treatment	Days after treatment, per cent germination							
		60 days		100 days		140 days		180 days	
		Tr.	Ck.	Tr.	Ck.	Tr.	Ck.	Tr.	Ck.
Large	Nov. 7	0	0	38	8	40	14	40	14
	Nov. 30	52	0	65	26	65	33	70	33
	Dec. 20	43	14	54	16	54	17	54	38
	Jan. 16	68	14	90	30	90	42	90	66
	Feb. 14	64	23	86	42	86	54	86	82
	Mar. 15	30	30	74	71	82	86	82	86
Medium	Nov. 7	0	0	0	0	12	12	31	31
	Nov. 30	8	0	16	0	22	10	22	18
	Dec. 20	0	0	4	4	50	18	82	27
	Jan. 16	38	0	72	20	84	27	84	43
	Feb. 14	39	9	86	20	86	35	86	50
	Mar. 15	8	8	62	40	98	98	98	98

planted previous to that time. But although the favorable effect of the treatment was less pronounced with Souvenir than with Remembrance, Table II shows distinctly earlier germinations for the treated lots. For

example, in columns 5 and 6, for the results at 100 days after planting, the treatments in November gave high or nearly complete germinations while the corresponding check lots showed no sprouts or very low percentages.

Alice Tiplady. The results with the variety Alice Tiplady are shown in Table III. The time of the year at which the test was made was an important factor in the response to the treatment. With the large size, in November and December the gains of treated over check lots were considerable, but maximum germination was not attained at the end of 180 days even by the treated lots. The January results showed that the cormels were losing their dormancy and the untreated lot gave 66 per cent germination in 180 days. The treated lot, however, showed 68 per cent germination in 60 days and 90 per cent in 180 days. In February and March the check lots were nearly out of the dormant period, the treated lots showing earlier germination but not a greater final percentage. A similar result was obtained with the medium size cormels except that it was not until March that the treated lot failed to show distinct gains over the check not only in rate of sprouting but also in the germination percentage which was reached in 180 days.

Gains in Time of Germination

Table IV shows for Souvenir and Alice Tiplady the number of days gained in the time of germination by the treatment. Columns 3 and 4 show the number of days required for 50 per cent germination in the case

TABLE IV
GAINS IN TIME OF GERMINATION OF GLADIOLUS CORMELS

Size of cormel	Date of treatment	Var. Souvenir			Var. Alice Tiplady		
		Days for 50% germ.		Gain, days	Days for 30% germ.		Gain, days
		Tr.	Ck.		Tr.	Ck.	
Large	Oct. 17	118	155	37	—	—	—
	Nov. 7	85	140	55	100	*	—
	Nov. 30	62	125	63	45	105	60
	Dec. 20	57	105	48	47	140	93
	Jan. 16	65	70	5	40	110	70
	Feb. 14	58	58	0	39	85	46
	Mar. 15	—	—	—	42	60	18
Medium	Oct. 17	150	168	18	—	—	—
	Nov. 7	87	168	81	180	180	0
	Nov. 30	75	137	62	*	*	—
	Dec. 20	65	112	57	120	180	60
	Jan. 16	65	78	13	58	150	92
	Feb. 14	67	80	13	55	130	75
	Mar. 15	—	—	—	75	92	17

* 30 per cent germination not reached in 180 days.

of Souvenir, and columns 6 and 7 show the time for 30 per cent germination in the case of Alice Tiplady. Columns 5 and 8 show the number of days gained by the treatment. The table shows that gains of about 60 to 90 days were obtained with these two varieties. It will be seen that the

TABLE V
TREATMENT OF VERY SMALL CORNELS OF VARIETIES ALICE
TIPLADY AND AMERICA

Variety	Cc. per l.	Duration of treatment, days					
		1	2	3	4	6	7
Alice Tiplady	7	71 69	57 73	* 12	58 61	83 42	81 36
	6	103 55	70 69	57 68	59 62	* 3	* 23
	4	102 43	73 55	57 61	58 68	77 41	125 37
	2	84 55	76 60	57 73	58 64	59 50	116 37
	1	130 36	73 66	61 67	61 54	62 68	58 59
	0	* 14	* 7	* 17	* 20	* 13	* 20
America	7	146 15	121 16	91 36	62 31	112 25	121 20
	6	* 4	146 15	61 33	65 44	* 7	109 17
	4	* 10	* 17	84 23	62 35	107 21	109 22
	2	* 12	* 12	62 35	67 36	95 22	74 30
	1	* 8	87 20	61 34	58 44	85 32	61 29
	0	* 6	* 9	* 0	* 11	* 10	* 7

Note: Ordinary type numbers show days required for 30 per cent germination in case of Alice Tiplady and 15 per cent germination in case of America; italics show per cent germination after 200 days; * indicates less than 30 per cent (Tiplady) or 15 percent (America) germination after 200 days.

amount of gain was largest not in the earliest tests but usually at a later period, being at a maximum in November for Souvenir and in December or January for Alice Tiplady. The lower values for days gained with the earlier treatments were due to the greater dormancy of the cornels, and in the later periods to their lack of dormancy.

The computations which are shown in Table IV for Souvenir and Alice Tiplady could not be made for Remembrance since, as shown in Table I, the germination of the untreated cormels was too low. If the time required for a germination percentage as low as 15 had been adopted for the comparison, the untreated lots would have reached this value in less than half of the tests made. For Remembrance cormels it is seen from Table I that the gain due to treatment was at least more than 120 days in all but five comparisons, and in these cases the gains were about 80 to 100 days.

TABLE VI
TREATMENT OF VERY SMALL CORMELS OF VARIETY HALLEY

Treatment		Dates on which treatments were made							
Cc. per l.	Duration, days	Oct. 4	Oct. 16	Nov. 18	Dec. 13	Jan. 16	Feb. 14	Mar. 15	Apr. 15
7	7	* 0	* 1	* 3	* 8	104 21	* 0	* 12	* 0
5	5	* 0	* 9	158 17	* 6	119 19	121 26	102 34	60 45
3	3	* 0	* 7	* 7	132 23	89 34	91 35	108 24	69 40
0	—	* 0	* 0	* 0	* 2	* 1	* 0	* 4	* 0

Note: Ordinary type numbers show days required for 15 per cent germination; italics show per cent germination after 180 days; * indicates less than 15 per cent germination after 180 days.

VERY SMALL CORMELS

The results of the treatment of very small cormels are shown in Table V and VI. Cormels of this size were very dormant as shown by the data for the untreated lots, the germination after 180 to 200 days being less than 25 per cent for Alice Tiplady, less than 15 per cent for America, and less than 5 per cent for Halley. The treatments with ethylene chlorhydrin, however, were effective in hastening the germination and in increasing the percentage of germination. The germination reached 60 to 70 per cent for Alice Tiplady, and 30 to 40 per cent for America and Halley. The time required for 30 per cent germination was about 60 days for Alice Tiplady, and for 15 per cent germination it was about 60 days for America and about 100 days for Halley.

In Table V are shown the results obtained by varying the amount of chemical and the period of exposure. This test was made late in January 1933. It is seen that the range of concentration and period of exposure needed for successful germination were not narrow, 1 to 6 cc. per liter for three to four days giving favorable results for both varieties in rate and

percentage of germination. Even a two-day treatment was satisfactory with Alice Tiplady cormels, but with America at least three days of exposure were necessary.

In Table VI are shown the results with very small cormels of Halley. The importance of the time of the year at which the treatment is applied is clearly shown. Thus, no treatment was effective until after December, but from January 16 to April 15 successful germination was obtained with either 5 cc. per liter for five days or 3 cc. per liter for three days. And, although even the most favorable treatments produced not higher than 45 per cent germination within 180 days from time of planting, in view of the very low germinations shown by the check lots, the results may be regarded as successful and as furnishing convincing evidence of the effectiveness of the chemical vapors in hastening the germination of very dormant cormels.

SUMMARY

Cormels of five different varieties of gladiolus, after being sorted into four different sizes, were treated with vapors of ethylene chlorhydrin at once after harvest and at intervals of three to four weeks thereafter until April or until untreated cormels gave good germination without treatment.

Sprouting of cormels was hastened by the use of 3 to 5 cc. of 40 per cent ethylene chlorhydrin per liter of air space within a closed container for a period of three to five days.

With varieties Souvenir and Alice Tiplady about 60 to 90 days were gained in the time required to reach a certain stage in germination (50 per cent for Souvenir and 30 per cent for Alice Tiplady). With Remembrance, although germination percentages as high as 50 or even 30 were seldom reached by untreated lots, it is estimated that gains in treated lots were about 100 to 180 days.

The amount of gain was largest not in the tests that were made immediately after harvest but at somewhat later periods, in November for Souvenir and Remembrance, and in December or January for Alice Tiplady. The lower values for days gained in the earlier treatment were due to the greater dormancy of the cormels and in the later period to their lack of dormancy.

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EFFECTS OF CHLORINATED WATER ON LAND PLANTS, AQUATIC PLANTS, AND GOLDFISH

P. W. ZIMMERMAN AND ROBERT O. BERG

The question of toxicity of chlorinated water on growing plants arises frequently among plant culturists and those conducting experimental work. Chlorine is known to be destructive to microorganisms and for that reason is used to purify the city water supplies. The practice of treating water calls for enough chlorine so that after it reacts with the organic matter present, 0.02 to 0.05 part of free chlorine per million of water will persist for 10 to 30 minutes (1, 7). Fluctuations in the chlorine demand of the water occasioned by the variation of organic impurities sometimes causes a chlorine residual as high as 1.5 p.p.m. when the water reaches the consumer. Daily tests at the Boyce Thompson Institute during July, August, and September, 1933, revealed that the tap water frequently contained 0.5 p.p.m. of free chlorine, occasionally 1.0 p.p.m., and rarely 1.5 p.p.m. The water tested highest during and immediately following rainy periods when the impurities in the water were increased or after the inception of a few cases of typhoid fever, during which times the chlorine dosage was increased.

Heinsohn (3) detected no injury to coleus, geranium, peach seedlings, or snapdragons watered with solutions having 2, 5, and 10 parts of chlorine per million of water. He noted, however, that a 1000 p.p.m. chlorine solution burned the edges of coleus leaves and the tips of leaves of peach seedlings and snapdragons. The number of applications required to produce the injury was not mentioned. Krone and Weinard (4) grew seedlings of celosia, gaillardia, pentstemon, petunia, phlox, verbena, and zinnia in a composted loam soil in two and one-quarter inch pots and watered them with cistern water containing 0, 5, 10, 50, 100, and 500 parts of chlorine per million of water. At the end of 30 days all the plants except the checks and those treated with the 5 p.p.m. solution showed injury in some degree. The weights and heights of the 5 p.p.m. group were less than those of the controls, and the weights and heights of the other groups decreased irregularly as the concentration of chlorine increased. Plants adjacent to pots watered with 1100 and 2200 p.p.m. chlorine solutions were burned by fumes emanating from the treated pots, but foliage near a pot treated with 500 p.p.m. solution was little affected.

Experiments were conducted at Boyce Thompson Institute during the summer of 1933 in order to determine the minimum injurious concentration of free chlorine in water, the character and extent of injury from the various concentrations, and the relative tolerances of different species of

land plants, aquatic plants, and goldfish. This paper reports the results of those experiments.

MATERIALS AND METHODS

Pure anhydrous chlorine obtained from the Hooker Electrochemical Company in the liquid state in a steel cylinder was used to chlorinate water for the experiments. The gas was passed into the water through a glass tube. The water was tested for free chlorine during the chlorination, and more chlorine or water added as required to bring the solution to the required chlorine content. Low concentrations were prepared by dilution from strong solutions of known chlorine content, and then tested to prove their concentration. The tap water used to prepare chlorinated water and to water the control groups usually tested from 0.2 to 0.5 p.p.m. of chlorine.

The sodium thiosulphate method of titration was used to standardize chlorinated water with 3 p.p.m. to 1000 p.p.m. of chlorine, while the orthotolidin method was employed to standardize water with less than 3 p.p.m. of chlorine. The iodimetric sodium thiosulphate titration was performed as described by Treadwell (6, p. 551-558). Freshly made 0.5 per cent Lintner starch solution, c.p. potassium iodide, and a standardized 0.01 normal sodium thiosulphate solution were the reagents employed. Chlorine water samples of 100 cc. were taken for the titrations. From the equation, 1000 cc. N/10 sodium thiosulphate solution = 3.546 g. chlorine (1, 6), it results that the number of p.p.m. of chlorine required in a solution, divided by 3.546, equals the number of cc. of 1/100 N sodium thiosulphate required to decolorize 100 cc. of a solution of the required chlorine content after an excess of potassium iodide and starch solution has been added.

The colorimetric orthotolidin test is accepted as more nearly accurate than the sodium thiosulphate titration for the quantitative determination of small amounts of chlorine. For this reason the orthotolidin method was employed for testing solutions less than 3.0 p.p.m. of free chlorine. Reagents and color standards were prepared and the test performed according to the methods of Enslow (2, p. 20-22). When the maximum color development was reached after the addition of the reagent to water samples, this color was compared with that of liquid standards in similar sample bottles, and the chlorine concentration of the sample thus determined directly in parts of chlorine per million of water.

Chlorinated water was stored in glass bottles and flasks that were filled to the top and tightly stoppered. Early experiments showed that the chlorine went out of solution rapidly when the water was stored in the laboratory, since the gas is most stable in solution at a low temperature in the dark (5). Therefore, the chlorinated water was taken to a dark cold room, having an average temperature of 10° C., immediately after it was

made up to the required concentration. Tap water for the control groups also was stored in the same cold room. Before each application the water was tested, and corrected if necessary to the required chlorine concentration.

All potted plants were watered once a day with water drawn from standardized supplies immediately before application. On Sundays the plants were watered with tap water.

EXPERIMENTAL RESULTS

Effects on potted plants. The following 13 species of plants were grown for treatment in composted loam soil in clay pots: oat (*Avena sativa* L. var. Clydesdale), coleus (*Coleus blumei* Benth.), calliopsis (*Coreopsis tinctoria* Nutt.), common cosmos (*Cosmos bipinnatus* Cav.), sulphur cosmos (*Cosmos sulphureus* Cav.), buckwheat (*Fagopyrum esculentum* Moench.), soybean (*Glycine max* Merr. var. Biloxi), barley (*Hordeum vulgare* L.), tomato (*Lycopersicon esculentum* Mill. var. Bonny Best), tobacco (*Nicotiana tabacum* L. var. Burley), rye (*Secale cereale* L.), African marigold (*Tagetes erecta* L.), and wheat (*Triticum aestivum* L.).

The different species were not all of the same age or size when the treatments began. Soybean was grown in four-inch pots and thinned to six plants per pot when the seedlings appeared above ground, while the grains were grown 11 per four-inch pot. Coleus, sulphur cosmos, tobacco, and tomato were three to six inches high when treatments with chlorinated water began. Calliopsis, common cosmos, and marigold were one to two inches high in two-inch pots when first treated. An experimental group usually consisted of four, five, eight, or ten plants of a variety. The numbers of pots in all groups of an experiment were always equal.

In the first experiments ten pots each of barley, buckwheat, oat, rye, and wheat were grown in loam soil and watered daily with water having one, three, five, seven, and ten parts of chlorine per million of water. After three weeks the plants showed no injury from any of the treatments. The next experiments were conducted with five groups of plants, having eight pots each of the grains, buckwheat, oat, and wheat, treated with 50, 100, 150, and 200 p.p.m. of chlorine. There was no injury from any treatment except from the 200 p.p.m., which spotted and bleached the leaves of the newly emerged seedlings. The injury progressed no further, after three weeks of treatment all four treated groups being equal in size and appearance with controls.

Buckwheat watered with 500 p.p.m. and 1000 p.p.m. of chlorine solutions germinated more slowly than that watered with tap water. Seedlings of the 500 p.p.m. group emerged one day later, and those in the 1000 p.p.m. group three days later than the controls. Both treated groups were deformed and yellowed or browned when they emerged. Nearly all plants in

the 500 p.p.m. group were dead seven days after they appeared above the soil, while most of those in the 1000 p.p.m. group were dead five days after emerging. The seedlings wilted and burned progressively and finally died.

In order to find out what effects high concentrations of chlorine in water would have on established plants, three groups of plants were watered with tap water, 500 p.p.m. chlorine water, and 1000 p.p.m. chlorine water, respectively. Each group contained five pots each of coleus, sulphur cosmos, oat, rye, and wheat, averaging six inches in height. The lower leaves on coleus were wilted six hours after the first watering with 500 p.p.m. solution. They browned during the succeeding days and fell off after a week. Meanwhile the middle and upper leaves showed similar injury, but it progressed more slowly. Roots as well as tops were browned. Lower leaves of cosmos showed marked epinasty after two treatments. The middle leaves developed a stippling of yellow or brown spots while the lower leaves yellowed or browned generally. After two weeks cosmos in both treated groups were still alive but roots and tops were burned badly. The three grains showed bleaching and wilting after the first application of both concentrations. During the first week leaves wilted, bleached, and browned progressively from oldest to youngest. Some plants were dead a week after the first treatment, and many more, but not all, died during the second week. Injury from the 1000 p.p.m. solution always was more serious and more rapid in development than that from 500 p.p.m. Untreated plants within a radius of one foot from those watered with 500 and 1000 p.p.m. solutions were wilted, bleached, and browned. This must have been due to the chlorine evolving from the chlorinated water. Krone and Weirard (4) noted similar effects from higher concentrations.

Experiments thus far described were conducted in open outdoor cold frames. Duplicate experiments with buckwheat and new experiments with other species were carried on in the greenhouse to find whether the plants would respond differently to daily watering with chlorinated water. While 100 p.p.m. and 150 p.p.m. did not noticeably injure buckwheat grown outdoors and 200 p.p.m. injured it but slightly, the same concentrations applied to plants in the greenhouse produced noticeable chlorosis and the last concentration, some stunting of growth. This difference may have been due to the more succulent growth of the plants under glass, or to the difference in air currents in the greenhouse and outside.

To determine more definitely the influence of a confined atmosphere two series of buckwheat, calliopsis, common cosmos, marigold, tobacco, and tomato were treated in the greenhouse as follows: one series grown on the open bench and the second under bell jars were both watered daily with water having 5, 10, 50, 100, 200, and 300 p.p.m. of chlorine. After three weeks of treatment none of the plants watered with 50 p.p.m. or less were injured. Those watered with 100, 200, and 300 p.p.m. were injured

both under bell jars and on the open bench. However, those under bell jars showed signs of injury first and the final amount of injury was greater than on the plants of the open bench.

Syringing with water during warm sunny weather is a common practice in the culture of a number of greenhouse crops. For this reason it was thought that injury sometimes attributed to chlorinated water by greenhouse men might be due to a combination of watering and syringing with chlorinated water. In order to determine how serious injury from syringing and watering at the same time can be, two series of buckwheat, calliopsis, common cosmos, marigold, tobacco, and tomato were arranged and treated exactly like those described in the preceding paragraph, except that in this case the tops of the plants were syringed with the same kind of water used in watering the soil. The four series were run simultaneously. As in previous experiments no injury resulted where plants were watered and syringed with 50 p.p.m. or less of chlorine. When water containing 100 p.p.m. or more was used for both syringing and watering the soil, the injury was greater than that produced by watering alone. Also, plants syringed and watered with these high concentrations under bell jars showed more injury than those on the open bench. The character of injury from syringing was somewhat different from that due to watering alone. Watering with higher concentrations of chlorinated water produced a general interveinal chlorosis. Syringing, however, produced a spotty chlorosis, the pattern of which depended on the distribution of the water droplets on the leaf. Syringing buckwheat, tobacco, and tomato with 100 p.p.m. produced slightly chlorotic spots. The 200 p.p.m. solution produced pale bleached spots, while the 300 p.p.m. decomposed irregular areas on the lower leaves, giving them a scalded appearance.

While water having a chlorine concentration of 50 p.p.m. or less never injured plants when applied in the ways and under the conditions so far described, there was still the possibility that injury might be produced by the same concentrations in a soil of coarser texture having less organic matter. To test this 20 plants each of buckwheat and soybean were grown in a composted loam soil (pH 4.8), an equal number in bank sand (pH 6.9) having almost no organic matter, and a third equal series in a mixture of equal parts of the loam and bank sand. Each series was then divided into four groups of five pots each and each group watered with a different concentration of chlorinated water. The strengths used were tap water, 5 p.p.m., 50 p.p.m., and 100 p.p.m. Watering began as soon as the seeds were planted and continued for three weeks. Both buckwheat and soybean in loam soil grew equally well in all treatments with the possible exception of slight retardation of growth where 100 p.p.m. of chlorine was used (Fig. 1 A). Where a mixture of sand and loam was used instead of loam soil alone, plants were slightly retarded from 50 p.p.m. of chlorine but not with

5 p.p.m. This shows that with smaller amounts of organic matter in the soil greater injury will result from a given concentration of free chlorine in the water. Figure 1 B illustrates the comparative ineffectiveness of bank sand and for preventing toxicity of chlorine on growth of soybean. Similar results were obtained with buckwheat and soybean. From the appearance of

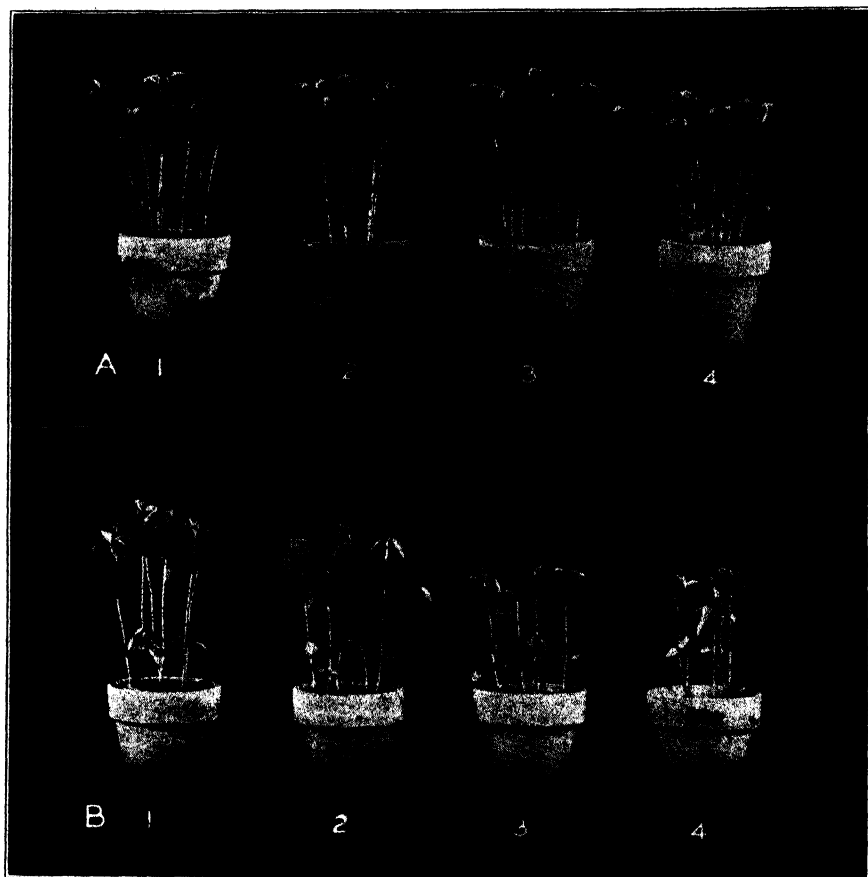


FIGURE 1. A. Soybeans grown in loam soil 17 days and watered daily with chlorinated water. (1) Check, (2) 5 p.p.m., (3) 50 p.p.m., (4) 100 p.p.m. B. Soybeans grown in bank sand 17 days and watered daily with chlorinated water. (1) Check, (2) 5 p.p.m., (3) 50 p.p.m., (4) 100 p.p.m.

the plants it was evident that in sand even the 5 p.p.m. retarded growth to some extent. Roots growing in sand watered with 5 p.p.m. were slightly browned, those in 50 p.p.m. browned and less extensively developed, while those in 100 p.p.m. were badly browned and very poorly developed. There was no evidence of injury to the roots from chlorine in the loam soil or in

the mixture of loam and sand. The chlorinated water had no appreciable effect on the acidity of the sand medium over a period of three weeks.

Chlorinated water having 500 or 1000 p.p.m. chlorine burned the roots of plants growing in loam soil, but concentrations of 300 p.p.m. or less had no noticeable effect on root growth in loam soil or in a mixture of sand and loam. Roots continued to grow well until the tops of the plants were badly injured. In contrast with the bad effects of low concentrations in sand, the soil high in organic matter neutralized or prevented 200 p.p.m. of chlorine from injuring the roots.

Effects on root growth from tomato cuttings. In order to ascertain whether



FIGURE 2. Root growth of tomato cuttings in chlorinated water after ten days. (1) Control (tap water), (2) 5 p.p.m. of chlorine in water, (3) 10 p.p.m., (4) 50 p.p.m., (5) 100 p.p.m.

roots would develop normally from tomato cuttings in water of lower chlorine concentrations the following experiment was conducted in duplicate in the greenhouse. Glass cylinders were capped with paraffined paper and a young tomato cutting was inserted through a center hole in each cap. The cylinders were then filled with chlorinated water as follows: 5, 10, 50, or 100 p.p.m. of chlorine. A glass tube inserted into each cylinder made it possible to draw out the old water and replace it with fresh chlorinated water daily. Figure 2 shows the results of the experiment. Roots developed first in the check and 5 p.p.m. solution and correspondingly later in the others as the chlorine concentration increased. The roots were comparatively short in 10 p.p.m. while in 50 and 100 p.p.m. solutions the roots grew only near the surface of the water. As can be seen in Figure 2 the

higher concentrations of chlorine bleached the stems and killed irregular areas of tissue. When water was tested 20 hours after being placed in the cylinders, it was found that the amount of chlorine remaining in solution varied from one-tenth to one-third of the original chlorine. This fluctuation may have been due to variations in illumination and temperature. When the same experiment was run with cylinders covered with black paper the chlorine remaining in solution was always greater than that in comparable uncovered cylinders.

Effects of chlorine on cut flowers. The question of whether chlorinated water impairs the keeping qualities of cut flowers is frequently raised. In order to answer this question cut flowers of snapdragon (*Antirrhinum majus* L.), *Gerbera* sp., *Gladiolus* sp., and *Rosa* (hybrid teas) were placed in solutions of chlorine as follows: 5, 10, 50, 100, 200, 300, and 500 p.p.m. The 5 p.p.m. and 10 p.p.m. solutions had practically no effect on the flowers. Fifty parts per million usually shortened the span of usefulness of gerberas and snapdragons but had practically no effect on the gladiolus or roses. Since tap water has never more than 1.5 p.p.m. it is safe to conclude that such chlorinated water is not apt to interfere with the lasting qualities of cut flowers. There may exist, however, some very sensitive species which should be tested.

Effects on goldfish and aquatic plants. Fish fanciers have sometimes expressed the belief that the chlorine in city water may be injurious to goldfish and aquatic plants. A few experiments were tried in an effort to throw some light on this problem. Six battery jars were kept on the laboratory table at the same temperature and away from sunlight. One jar, the check, contained three liters of tap water that had been standing in the open for at least 24 hours so that the chlorine content was always near zero. The other five jars contained three liters of a solution having 0.5, 1.0, 1.5, 2.0, or 3.0 p.p.m. of chlorine. Each jar contained eight sprays of *Cabomba caroliniana* Gray, four sprays of *Elodea canadensis* Michx., and one of each of the following goldfish, *Carassius carassius* L. varieties Fantail, Common, and Shubunkin, all roughly comparable in size and appearance. Water was replaced daily by fresh chlorinated water of the required strength and of equal temperature. Fish that died were replaced in kind before the next renewal of water, because it was found in preliminary experiments that when there are fewer fish in a container they succumb to chlorinated water more easily. Over a period of six days three fish died in the 3 p.p.m., one in the 2 p.p.m., and none in any of the other jars. At this time none of the aquatic plants showed any injury except in the 3 p.p.m. solution, where both *Cabomba* and *Elodea* were slightly chlorotic. It was found that the chlorine concentration fell rapidly with the aquatic plants present; for example, the water that originally had had 3 p.p.m. of chlorine had only 0.2 p.p.m. after three hours, while the 2 p.p.m. dropped to 0.1

p.p.m. in the same time. The plants were removed from the jars to find whether they had counteracted the toxic effect of the chlorinated water on fish. At the same time, the 3 p.p.m. treatment was discontinued because it appeared to be toxic to some degree. During the succeeding eight days four fish died in the 2 p.p.m., two in the 1.5 p.p.m., and none in the other jars. This may indicate that the water plants had inhibited the toxic action of the chlorinated water during the first six days, but variations in the rate of loss of chlorine made it impossible to show by tests that the plants removed some chlorine from the water and thus protected the fish. The small number of fish used and a number of variable influencing factors make it unwise to draw any final conclusions, though it seems likely that 1.5 p.p.m. without the presence of plants was toxic to goldfish.

Of a total of ten fish killed during 14 days, five were Fantails, three of the Common variety, and two Shubunkins. The original numbers of each variety in all treatments were equal. In this and in other experiments, Fantails usually succumbed to the action of chlorinated water more rapidly than Common or Shubunkin goldfish. It appeared also that the Shubunkin goldfish were more resistant than the Common variety.

It was desired to compare the effects of a constant renewal of water to keep the chlorine content constant with the effect of renewing water only once a day. In the first treatment the chlorine concentration remains nearly constant while in the second it falls rapidly as has been shown. In five experiments one of two jars had a steady inflow of chlorinated water at the rate of about four liters per hour. The second jar, a check on the first, did not have the water replaced after the initial filling from the same source. Both jars had equal numbers of comparable fish and the experiments were run for from three to eight hours. The constantly renewed water killed fish at as low a concentration as 1.0 and 1.5 p.p.m. in eight hours, while fish in the check jar were normal the next day. This may indicate that where chlorinated water flows into the container constantly there is likely to be injury from a lower chlorine concentration than where the water is renewed only once a day.

Cabomba and *Elodea* showed some chlorosis from 3 p.p.m. renewed daily after a week of treatment. Two days in 5 p.p.m. bleached both species badly, but when the sprays were placed in tap water the terminal buds unfolded fresh green leaves after a few days. Five days in 5 p.p.m. killed both species so that they could not be revived.

SUMMARY

1. None of the species of plants grown in loam soil and watered, syringed, or watered and syringed with chlorinated water having 50 p.p.m. or less of chlorine in water, were injured or retarded when grown in pots in cold frames, on open benches in the greenhouse, or under bell jars in the

greenhouse. Chlorine concentrations of 100 and 150 p.p.m. injured or retarded some plants but had no effect on others. Concentrations of 200 and 300 p.p.m. always produced some degree of injury to the tops but had no appreciable effect on roots. Concentrations of 500 and 1000 p.p.m. retarded the emergence of seedlings, burned and usually eventually killed tops and roots of plants.

2. A combination of watering and syringing generally produced greater and more rapid injury than watering the soil alone. Syringing the tops usually was somewhat less injurious than watering the soil with the same solutions.

3. Injury from all treatments was in most cases more serious and more rapid in the greenhouse than in open cold frames, and worse under bell jars in the greenhouse than on the open greenhouse bench.

4. Tops of plants grown in a mixture of equal parts of loam and sand were retarded in top growth by solutions of 50 p.p.m. and 100 p.p.m. but not by 5 p.p.m. Roots were never injured in this soil. Plants grown in sand were retarded in root and top development by solutions of 5, 50, and 100 p.p.m. chlorine, the degree being somewhat in relation to the concentration of chlorine in the solution.

5. Chlorinated water applied to sand and to loam soil for three weeks had no significant effect on acidity of the media.

6. Roots from tomato cuttings in chlorinated water were retarded in size directly as the chlorine concentration increased from 10 p.p.m. but they were not affected with 5 p.p.m.

7. Cut flowers were not affected with free chlorine in water up to 10 p.p.m. Fifty parts per million injured gerbera and snapdragons but not gladiolus or roses.

8. Under the conditions stated in the text goldfish were killed by chlorine solutions of 1.5, 2.0, and 3.0 p.p.m. where the water was changed daily. Where water was constantly renewed, concentrations of 1.0 and 1.5 p.p.m. were toxic. Fantail variety appeared to be less resistant than the Common variety of goldfish, while the Common variety was less resistant than the Shubunkin variety.

9. Aquatic plants appeared to counteract in some degree the toxic effects of chlorinated water on fish. *Cabomba* and *Elodea* were discolored by chlorinated water containing 3 p.p.m. in one week when the water was refreshed daily. A concentration of 5 p.p.m. produced injury in two days and death in four days.

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CELLULAR CHANGES IN RING-SPOT¹

MARK W. WOODS²

Priode (9) made freehand sections of stem lesions of ring-spot in New Zealand spinach, but did not attempt to make a detailed microscopical study of them. The occurrence of intracellular bodies in lesions of ring-spot has been reported by the writer (14). To date there has been no published work describing the general cytological and histological changes that occur in the leaf as a result of lesional development. The present paper reports results of such observations.

Price (8) has given a general review of the more important literature of ring-spot. The literature dealing with cytological and histological studies of other virus diseases is very large. Goldstein (5) and Cook (1, 2) have presented excellent reviews of much of this.

MATERIALS AND METHODS

The methods of growing plants under the usual greenhouse conditions, and the collection of material therefrom, have already been described (14). The ring-spot disease worked with is that which was first definitely described by Fromme, Wingard, and Priode (4).

A comparative study of lesions formed under rigidly controlled environmental conditions was not attempted. Under the conditions obtaining in the various experiments certain types of lesions were regularly obtained. These are referred to as chlorotic, partly necrotic, completely necrotic, etc. Both living and preserved material were examined in these studies. In the latter case lesions were fixed in either Flemming's weaker solution or in formol-acetic-alcohol as previously described (14). Most of the material was stained with Flemming's triple stain, but safranin and light green and Heidenhain's iron-alum haematoxylin (with or without a counter-stain) were sometimes used. The methods of growing plants under special conditions are described elsewhere in this paper.

¹ By permission of the Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, an investigation on ring-spot, which was begun there in the summer of 1931, has been carried on in the Department of Botany at the University of Maryland in partial fulfillment of the requirements for the degree of Master of Science. This paper represents part of the thesis requirements.

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LESIONS FORMED ON NICOTIANA TABACUM L. VAR. TURKISH

PRIMARY LESIONS

In Turkish tobacco the lesions that developed on inoculated leaves during the months of June, July, and August in the greenhouse were typically much less necrotic than those produced during the fall and winter months. In the summer, primary lesions of ring-spot appeared in from two to four days after inoculation of vigorous young Turkish tobacco plants. Such lesions were usually circular varying from slightly less than one millimeter to two millimeters in diameter. They generally consisted of a narrow necrotic ring surrounding a normal-appearing central island of tissue. The latter sometimes contained a central necrotic spot only a few cells in diameter. Occasionally, however, the whole first-visible lesion was completely necrotic. In either case alternating bands or fine rings of normal-appearing and necrotic or chlorotic tissue developed around the center of the lesion. Typical primary summer lesions of ring-spot are shown in Figure 1 A, B, and C.

It was desired to observe microscopically as well as macroscopically various stages in the development of primary lesions in the living leaf. Several leaves on each of a number of vigorous young Turkish tobacco plants were therefore inoculated with ring-spot virus on June 13. The plants were grown in the greenhouse until June 16 when the first lesions were observed. On the latter date three plants were removed from the greenhouse to a south window of the laboratory where they were grown during the rest of the experiment.

The plants in the greenhouse made the most rapid growth and developed the most severe necrotic symptoms. The latter seemed to be due to the rapid desiccation of diseased tissues that occurred in the greenhouse. Typical summer lesions developed on the plants removed to the laboratory. These, though less necrotic, were essentially like those formed in the greenhouse, and were very desirable for study. Five primary lesions on the plants in the laboratory were selected for detailed study.

Three of the lesions studied are shown in Figure 1 A, B, and C. The photographs were made nine days after inoculation at the conclusion of the experiment. The first visibly affected areas were not observed early enough to warrant any conclusions as to their mode of formation. The development of the secondary, tertiary, etc., necrotic areas were therefore studied in detail. The sequence of events leading up to visible necrosis appeared to be much the same in the different lesions studied.

Apparently during the night changes took place in the cells of the mesophyll that resulted in the formation of visibly necrotic areas in the leaf. Such areas were not visible until midnight or later. At first they were very indistinct, generally not becoming clearly visible until the middle of the

day. Macroscopically such areas appeared water-soaked in the early stages of breakdown, varying in color from green to grayish-green, and were much more in evidence by transmitted than by reflected light. Cell breakdown seemed to be limited almost entirely to the mesophyll. The palisade layer was generally the first affected, the spongy layer breaking down considerably later or not at all. In some instances, however, the spongy layer was the first to become necrosed.

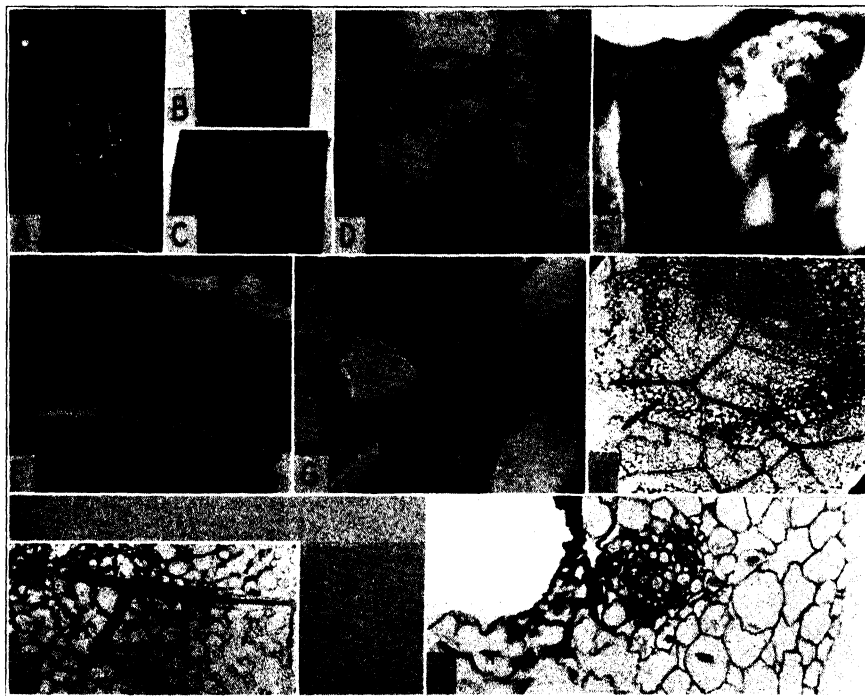


FIGURE 1. Primary lesions of ring-spot in Turkish tobacco. A, B, C, summer lesions nine days after inoculation; D, cells in a lesioned area four days after inoculation, the plastids showing starch retention, $\times 600$; E, necrotic palisade cells in a "summer" lesion three days after inoculation, $\times 700$; F, necrotic palisade cell containing numerous starch grains, three days after inoculation, $\times 400$; G, section through periphery of a necrotic area of a "winter" lesion showing abrupt transition from necrotic to nearly normal cells, four days after inoculation, $\times 400$; H, tangential section of a "summer" lesion four days after inoculation, $\times 20$; I, tangential section of periphery of a "winter" lesion four days after inoculation, $\times 53$; J, section through a necrotic area adjacent to a secondary vein in a "winter" lesion seven days after inoculation, $\times 105$.

In early stages of necrosis water lost by the affected cells had gone into the intercellular spaces giving the tissue a translucent or water-soaked appearance. This loss of water from the protoplasts of affected cells appeared

to be accompanied by degeneration of the cytoplasm, and usually the chloroplasts as well. In some cells, however, disintegration of the chloroplasts apparently did not occur.

Drying of the affected areas usually proceeded rapidly. By the middle of the day on which necrosis was first visible the affected cells were generally completely collapsed, leaving a marked depression in the lamina. Sometimes, however, the loss of water from the diseased cells must have proceeded slowly. In certain instances the first stages of necrosis did not become visible until about noon, later stages of necrosis not appearing until evening. In any case, drying of the affected tissues was accompanied by a transition from the water-soaked grayish-green or whitish-green appearance to a brownish-white or brown color. The epidermal and guard cells often remained turgid considerably after the underlying mesophyll had collapsed. The necrotic areas occasionally enlarged somewhat during the drying out process, but as a rule the greater portion of these regions formed at about the same time.

On plants grown in the greenhouse the general sequence of events in the breakdown of the mesophyll was essentially the same as that encountered in the laboratory plants. The chief differences were a more rapid drying out and collapse of the cells in the affected areas of the greenhouse plants. In either case those events immediately preceding visible breakdown in the leaf seemed to have occurred at night. During the following daylight hours necrotic areas were merely accentuated by drying out and collapse of the diseased cells.

Cook (3) in reporting studies of a spotting of mosaic-diseased tobacco leaves in Porto Rico observed that the affected cells appeared to break down during the night, later drying out and collapsing. The general sequence of events in the formation of necrotic areas in ring-spot is essentially like this. In ring-spot the number of necrotic rings formed is not necessarily equivalent to the number of periods of darkness, but there seems to be some correlation between the two. The fact that certain areas within a lesion may not become necrotic for many days indicates that some of the cells must develop a tolerance to the virus. It seems possible that those cells invaded during the day are more likely to remain alive than those invaded during the night. Much more information is needed on these points, however.

Further studies of primary lesions in Turkish tobacco were made in prepared material. All cytological and histological studies of primary lesions were made in relatively mature leaf tissue. The sequence of events in cell breakdown, as determined from studies in sectioned and stained material, appeared to be essentially the same in both summer and winter lesions. As already pointed out, however, necrosis was much more extensive in the latter. In either case the first sign of cell alteration observed

was an abnormal accumulation of starch in the plastids. The same phenomenon has been observed in mosaic-diseased tobacco leaves by Holmes (6), Samuel (10), and Woods (12, 13). Price (8) has observed that non-necrotic areas of ring-spot diseased leaves of Turkish tobacco may retain starch abnormally. Narasimhan (7, Fig. 2c) illustrates cells from spike-diseased sandal leaves that contain larger than normal starch grains in their chloroplasts.

In ring-spot material the chloroplasts of some of the diseased cells contained large starch grains that contrasted sharply with the smaller grains in chloroplasts of more normal cells. Many of the cells of the mesophyll thus affected appeared to be normal in other respects. Figure 1 D shows several cells from the periphery of a winter lesion four days after inoculation. The material was collected early in the morning and fixed in Flemming's weaker solution and stained with the triple stain. The plastids in cells outside of the lesioned area were apparently free of starch, those shown in the figure contained large starch grains. Some of the cells in the lesioned area displayed no symptom of disease other than the retention of starch.

Following the inhibition of starch hydrolysis the next visible sign of cell breakdown seemed to be a disintegration of the chloroplasts, followed or accompanied by alteration of the cytoplasm, and nuclear degeneration. In some cells only a few of the plastids were disintegrated before the cells dried out and collapsed, but in other instances nearly all of the plastids were broken down first. It was not possible to correlate definite stages of nuclear breakdown with particular stages or amounts of plastid disintegration.

Cells of the mesophyll in the early stages of breakdown contained a fine granular material that often extended into the vacuole. In later stages, this material more or less completely filled the lumen before drying out and collapse of the cell occurred. This granular material was apparently a product of plastid degeneration. In the early stages of breakdown it stained with the triple stain about as the normal plastids, but in more advanced stages it exhibited a much greater affinity for safranin.

In lesions fixed in Flemming's weaker solution, cells in early stages of breakdown often showed much better penetration of the fixing fluid than did normal cells. This was evidenced by the excellent fixation of the apparently normal nuclei in some of the diseased cells, whereas in the cells of the normal-appearing regions of the leaf the nuclei were very poorly preserved. Formol-acetic-alcohol, however, gave uniformly good fixation throughout.

Though not always the case, nuclear degeneration generally seemed to have occurred after destruction or severe modification of the cytoplasm. The nuclei of cells in the early stages of breakdown tended to stain more

heavily with the safranin than those in apparently normal cells, and were sometimes smaller than normal. In many instances, however, cells that contained much granular material with few recognizable plastids contained nuclei of apparently normal form and staining reaction.

In completely necrotic cells remnants of plastids, nucleus, and cytoplasm were often discernible, but in a much altered condition. In many instances, however, the necrotic cells formed an indiscriminate mass. After applying safranin and light green the walls of completely necrosed cells stained green as in normal cells, but the shrivelled remains of the protoplast stained bright red. Starch grains were often present in the remains of the protoplasts.

Figure 1 E shows three cells from the palisade parenchyma in a necrotic area of a primary lesion in Turkish tobacco three days after inoculation. The two cells on the left were completely necrotic whereas the one on the extreme right was in an intermediate stage of breakdown. Numerous large and small starch grains were visible in the completely necrotic cells. In one of them the remains of the nucleus can be seen.

Figure 1 F shows in the center a completely necrosed palisade cell in a primary lesion from Turkish tobacco three days after inoculation. Complete plastid disintegration had occurred in this cell. Numerous starch grains were present in the remains of the protoplast. This material was fixed in formol-acetic-alcohol and stained with safranin and light green. Starch grains were also readily demonstrated in the remains of such necrotic cells by staining untreated material with iodine-potassium iodide solution.

In both summer and winter primary lesions collected four to eight days after inoculation all of the above mentioned stages in cell breakdown were frequently observed. In summer lesions the central islands of macroscopically normal-appearing tissue contained many cells that appeared to be uninjured in any way. By studying lesions collected early in the morning, however, it could be seen that there was nearly always a region about three palisade cells in width near the visibly necrosed area in which the chloroplasts contained more starch than normal. In some lesions all, or nearly all, of the cells of the central island displayed this abnormality. In material collected in the afternoon the presence of starch in all cells of the mesophyll tended to mask this symptom. The palisade often seemed to be more severely affected than the spongy parenchyma.

A transitional zone, several palisade cells wide, often existed between the more or less normal-appearing cells in the center of the lesion and the completely necrotic portions. In this transitional area various intermediate stages in cell breakdown occurred. In some instances the transition was very abrupt, normal-appearing cells (except for abnormal amounts of starch in the plastids) lying adjacent to completely necrotic cells. Similar

transitional regions existed between the necrotic rings and green bands of tissue encircling the centers of the lesions. Intracellular bodies were seldom observed in lesions collected only four to eight days after inoculation. The occurrence and description of these inclusions have already been reported (14).

Primary winter lesions of ring-spot collected in December, four to seven days after inoculation, were studied. In all of the lesions observed there was a transitional zone between part or all of the peripheral necrotic region and normal-appearing areas of the leaf. This transitional zone was generally wider and more obvious than in summer lesions varying from one to about six palisade cells in width. In some places, however, the transition was very abrupt as shown in Figure 1 G.

In Turkish tobacco the veins of the leaf during summer or winter were much more resistant to necrosis than the mesophyll. In or very close to the necrotic regions, however, alteration of the veinal tissues sometimes occurred. In larger veins usually only the border parenchyma cells were affected. Small veins were often completely necrotic. A typical condition is illustrated in Figure 1 H. Macroscopically this lesion was characterized by the presence of two necrotic rings enclosing areas of apparently normal tissue. Most of the small veins, as can be seen in the figure, were necrosed where they crossed the necrotic areas. There seemed to be no tendency, however, for the necrosis to follow along the actual veinal tissues as sometimes occurred in certain other suscept.

In some lesions the necrotic areas tended to follow along the larger veins as shown in Figure 1 B and C. In such lesions, however, the breakdown was confined to the bordering mesophyll rather than to the veins themselves. Figure 1 J shows a portion of a winter lesion four days after inoculation, in which the veins included in the visibly lesioned area were necrosed to about the same extent as the adjacent mesophyll. In the central necrotic area, the veins formed an intensely staining, shrunken mass in which the remains of the vascular elements were distinguishable. In the transitional region the xylem elements of the veinlets appeared unaffected, but the bordering parenchyma cells generally contained partly disintegrated plastids and degenerate nuclei.

Though not usually the case, secondary veins were sometimes slightly necrosed in lesioned areas. Figure 1 K shows a cross section through a secondary vein in a primary winter lesion seven days after inoculation. The necrotic area for a considerable distance along this vein was not more than one parenchyma cell layer away from the vascular elements. In limited areas even a few of the phloem cells were necrotic.

Primary lesions of the summer type, 15 days or more after inoculation, were generally characterized by more extensive necrosis than in younger lesions. Areas one to three palisade cells wide, close to the completely

necrotic regions, often contained cells with fewer and smaller than normal chloroplasts in addition to granular debris. The cytoplasm in these cells was apparently not altered severely and the nuclei generally appeared to be uninjured. Breakdown in these transitional cells had apparently proceeded very gradually in contrast to the rapid breakdown that had obviously occurred in the formation of the first necrotic areas of the lesions.

In many cells the plastids were considerably disintegrated although the cytoplasm and nuclei appeared to be still intact. Sometimes the cytoplasm in the transitional cells appeared to be thickened. This may have been due, however, to an accumulation of some product of abnormal cell metabolism. The occurrence of this cytoplasmic thickening often seemed to be associated with the presence of intracellular bodies in the diseased cells. These inclusions were observed much more frequently in lesions several weeks old than in those collected only a few days after the inoculation.

SYSTEMIC LESIONS

The cytological pictures in systemic lesions in regard to disintegration of the protoplasts were generally essentially the same as in the primary lesions. Histologically, however, the various tissues of the leaf displayed certain peculiar modifications. The type of modification that occurred in most instances seemed to have been due to the age of the leaf at the time of the death of certain cells and the resultant change in the mechanical structure of the leaf. All of the abnormalities observed, however, cannot be attributed to mechanical changes alone.

From studies in a number of systemic lesions, collected at different times of the year, it was concluded that the cytological and histological pictures in summer and winter systemic lesions were essentially the same. Necrosis tended to be a little more extensive in the latter, however.

Portions of the mesophyll were often destroyed while the leaf was very young. Figure 2 B is a photomicrograph of a section cut through a young leaf 15 days after inoculation. This leaf was about one centimeter long, and was abnormally chlorotic. No necrotic areas were observed macroscopically. The distribution of necrotic cells in the mesophyll can be seen in the figure. These are darkly stained and more or less collapsed. Necrosis was limited to several narrow bands running across the lamina. Intracellular bodies were not observed in leaves of this age.

In certain areas of systemic lesions from older leaves, such as the leaf shown in Figure 2 A, necrosis was sometimes limited entirely to the palisade parenchyma. In many lesions examined the palisade must have been killed while the leaf was very young. Figure 2 E shows a section through a necrotic area of a systemic lesion 15 days after inoculation. The palisade and lower spongy parenchyma were extensively necrotic. This peculiar distribution of necrosis was commonly encountered in systemic lesions, the

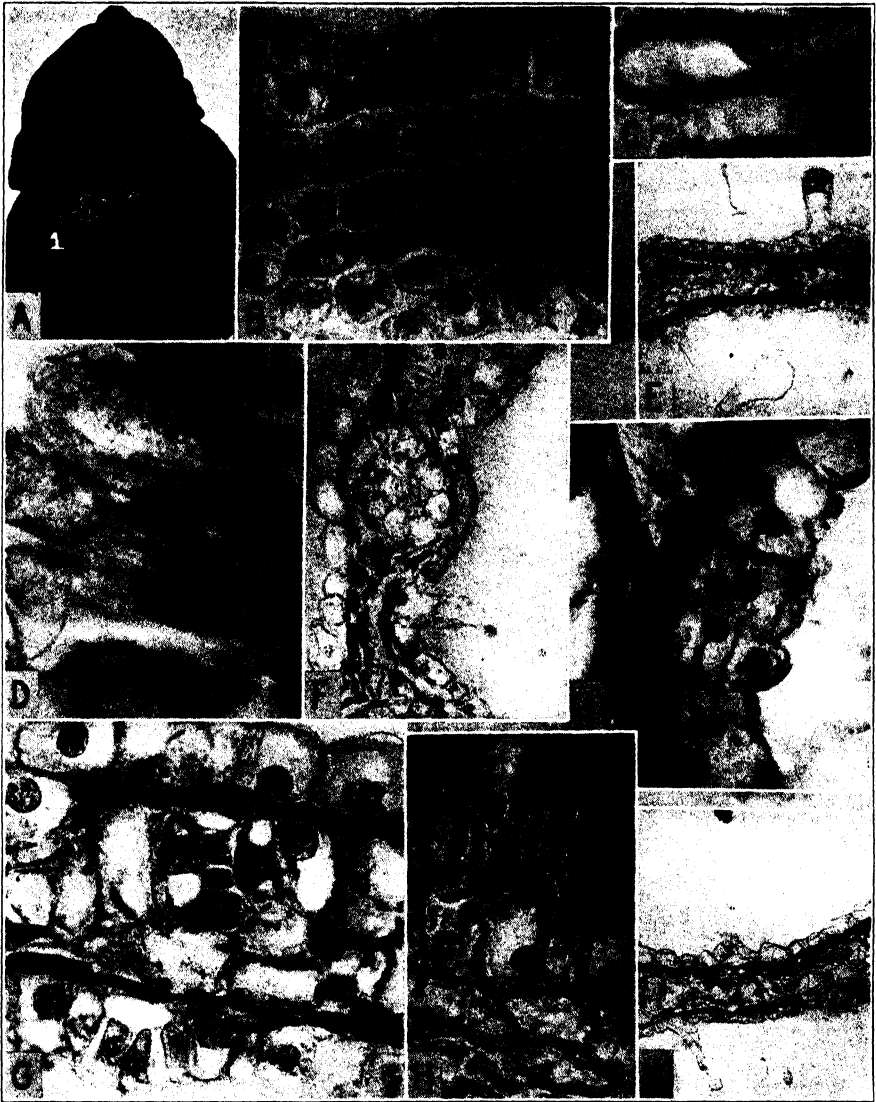


FIGURE 2. Systemic lesions of ring-spot in Turkish tobacco. A, 15 days after inoculation; B, section through a lesioned area of a young leaf, $\times 480$; C, palisade cells from a normal-appearing area of a systemic lesion 15 days after inoculation, $\times 840$; E, necrotic palisade and lower spongy parenchyma in a systemic lesion 15 days after inoculation, $\times 114$; F, necrotic areas in a lesion 15 days after inoculation. At one point the mesophyll is completely necrotic although the epidermal cells are apparently uninjured, $\times 222$; G, partially necrotic mesophyll in a lesion 15 days after inoculation, $\times 600$; H, normal-appearing area of same lesion, $\times 600$; J, activated epidermis over completely necrotic mesophyll 15 days after inoculation, $\times 510$; K, enlargement of certain cells of the mesophyll in a systemic lesion 15 days after inoculation, $\times 114$.

palisade being more often necrotic than the spongy layer. In either case the remaining cells of the mesophyll were likely, though not always, to show some plastid degeneration.

Figure 2 G shows a section through a necrotic area in another systemic lesion 15 days after inoculation. The palisade and lower spongy parenchyma were obviously killed while the leaf was very young. Some of the spongy parenchyma around a portion of the vein was also necrotic. The epidermis was apparently unaffected. A stoma can be seen opening directly into the necrotic area in the lower mesophyll. Figure 2 H shows a normal-appearing area of the same leaf. Figure 2 F shows a cross section of a necrotic area in lesion number one in Figure 2 A. This illustrates a condition frequently observed. The mesophyll in certain areas was completely necrotic, the epidermal cells and trichome being apparently uninjured.

The upper epidermal cells, remaining alive after all of the mesophyll had been killed, sometimes became active forming several layers of cells over part of the necrotic area. This condition was observed in several lesions. Figure 2 J shows two and three layers of cells derived from the upper epidermis over completely necrotic mesophyll in a systemic lesion 15 days after inoculation. The cells contained no plastids, and were apparently devoid of abnormal granular material.

In some lesions where portions of the mesophyll had become necrotic before the leaf was fully developed, a few abnormally large cells were observed. Figure 2 K shows a section through a lesion 15 days after inoculation. Certain cells of the spongy parenchyma were considerably enlarged, containing prominent nuclei. These cells contained very few plastids and but little granular debris. Some of them were nearly surrounded by necrotic tissue. Another type of cellular modification that sometimes occurred was a suppression in length of some of the palisade cells and in the development of intercellular space in the spongy parenchyma. These changes in the structure of the leaf were never very extensive and occurred only adjacent to necrotic areas. Woods (13) found that in the chlorotic areas of mosaic-diseased tobacco leaves the whole mesophyll was made up of more or less isodiametric cells with little intercellular space. Similar observations have since been made by numerous other workers. In ring-spot the cells are usually killed rather than merely retarded in their development.

Chlorotic areas such as those occurring in the systemic lesions shown in Figure 2 A contained cells in various stages of disintegration. Figure 2 D shows at the bottom portions of two palisade cells in which almost complete protoplasmic disintegration had occurred. In the two cells just above these the plastids were almost completely disintegrated, but the nuclei and cytoplasm were not visibly broken down. The two uppermost cells in the figure contained a few plastids that were still recognizable. Normal-appearing cells from a green area of the same lesion are shown in

Figure 2 C. The chief difference between these chlorotic cells and the transitional cells described under primary lesions was that plastid disintegration had generally proceeded farther in the former before collapse and drying out of the cells occurred. This seemed to be due to the greater persistence of the cytoplasm in the chlorotic cells of the systemic lesions.

In some of the chlorotic areas there seemed to have been a suppression of the number and size of the chloroplasts. In such cases the chloroplasts were about two-thirds the size of those in the normal-green portions. It was always difficult to determine, however, whether chlorosis was due either to plastid suppression, disintegration, or both. Cook has advanced the theory that tobacco mosaic virus inhibits the chloroplasts for a time. He states (1, p. 69), however, that his ". . . studies do not include a study of the lesions which frequently occur in connection with virus diseases of plants."

LESIONS FORMED IN CONTINUOUS DARKNESS

Price (8) has shown that ring-spot lesions produced on Turkish tobacco plants grown in continuous darkness consist of water-soaked areas or solid necrotic spots. In the present study results essentially in accord with those of Price were obtained. In one experiment one-half of each of three leaves on two Turkish tobacco plants were inoculated with ring-spot virus. One plant was placed in the laboratory under a bell jar from which all light was excluded, the air inside being kept saturated with moisture. The other plant was placed in a dark cabinet about one foot away in which the air was kept relatively dry by calcium chloride. Soil moisture was kept approximately the same in both cases. Both plants were thus subjected to practically the same conditions with the exception of humidity of the atmosphere.

Seven days after inoculation each of the inoculated halves of the leaves from the plant grown in dry atmosphere contained from one to four completely necrotic slightly brownish-gray lesions. The transition from completely necrotic to normal-appearing tissue was generally abrupt though some of the lesions appeared to be surrounded in part by a very faint chlorotic halo. The uninoculated halves of the leaves were normal in appearance.

The inoculated halves of the three leaves from the plant grown in water-saturated atmosphere contained lesions that were decidedly yellow-chlorotic. On the largest leaf inoculated several of the lesions had coalesced, the central portion of each being marked by a small gray necrotic spot.

The experiment was repeated some time later with essentially the same results. Material from both experiments was examined cytologically as in the preceding studies. Except for the complete lack of starch in any of the cells and the completeness of plastid breakdown, the lesions formed in

darkness in dry air resembled somewhat the typical primary winter lesions previously described.

Sections of lesions formed on plants in darkness in humid air presented quite a different aspect from those formed in dry air in darkness. The cells of the central chlorotic areas were not collapsed and were more or less filled with granular debris. The nuclei of these cells were shrunken and heavily stained, and the cytoplasm had apparently been disintegrated. In some of the cells numerous hyaline spheres that seemed unmistakably to be remnants of plastids were present. These hyaline spheres, which were about the size of chloroplasts, may be similar to the transparent vesicles observed by Sorokin (11) in mosaic-diseased tomato cells. She also found granular material in cells which were devoid of their normal contents. Cells outside of the chlorotic areas of the lesions formed in humid air in darkness did not show any signs of degeneration.

The primary lesions formed under the two sets of environmental conditions, just described, did not differ appreciably in size. In either case most of the chloroplasts were completely disintegrated before final collapse of the cells. There was some evidence, however, that plastid breakdown was slightly more extensive in those lesions formed in humid air. The hyaline spheres observed in the lesions from the plants grown in humid air probably do not constitute an important point of cytological difference between the two. Such structures have been observed in other experiments in primary lesions formed in continuous darkness in relatively dry air. All of the above mentioned cellular modifications were observed in lesions fixed in Flemming's weaker solution, as well as in formol-acetic-alcohol.

Both living and fixed material were examined from primary lesions formed on plants grown in continuous darkness in a dark chamber in the greenhouse as well as in another one located in a building near-by. The situation in these lesions, which were completely necrotic, was practically identical with that in lesions formed in dry air as already described. Intracellular bodies were not observed in any of the lesions formed in continuous darkness. It seems that these structures do not develop when the cells are very quickly killed.

LESIONS FORMED ON SUSCEPTS OTHER THAN TURKISH TOBACCO

Cytological and histological studies were made in primary lesions in *Nicotiana tabacum* L. var. Havana Seed-leaf, *N. rustica* L., *N. glutinosa* L., *N. glauca* R. Grah., and *Petunia* sp. These studies, while not as extensive as those made in Turkish tobacco, indicated that the stages in cell breakdown and the relative resistance of the different tissues to the virus were not essentially different from those observed in Turkish tobacco.

VIGNA SINENSIS ENDL. VAR. BLACK-EYE

In Black-eye cowpea the vascular tissues were particularly susceptible to the virus. This was in marked contrast to the other susceptibles. Vigorous young plants of this cowpea were nearly always readily infected with ring-spot by inoculating exactly as in the case of tobacco. Plants inoculated through the first simple leaves or the first compound leaves developed primary symptoms of ring-spot within three to five days. The primary lesions consisted of small brown or reddish-brown spots that rapidly enlarged. Lesions were always of the completely necrotic type whether formed in winter or summer.

Vascular tissues of the leaf were particularly susceptible, the necrosis often extending along the veins considerably in advance of the necrotic mesophyll. While some of the lesions were circular they more often tended to be quite irregular in shape. In primary lesions, observed three days after inoculation, the necrosis was confined mostly to the palisade. In a few days, however, all of the mesophyll and the epidermis became necrotic.

Systemic lesions developed several days after the appearance of primary symptoms of the disease. Unlike Turkish tobacco and many other susceptibles, the stem growing points were generally killed, the plants dying from the effects of the virus.

For cytological and histological study, lesions were fixed in Flemming's weaker solution and stained with the triple stain. This fixative gave fairly good results, but as with material from other susceptibles, nuclear preservation was often poor. All of the lesions studied were obtained from plants grown in the greenhouse during the month of July. Most of the studies were made in typical primary lesions collected three and five days after inoculation.

In general the stages in cellular breakdown of the mesophyll in Black-eye cowpea were like those already described in the other susceptibles. The action of the virus on the veinal tissues, however, was very different.

From studies in several lesions it was observed that in small veins radiating out from the necrotic area, the xylem elements were often stained very dark red and were considerably disintegrated though the border parenchyma and adjacent mesophyll were only slightly injured. Figure 3 A is a photomicrograph of a section cut tangentially through a portion of a lesioned area collected three days after inoculation. The small branch of the normal-appearing vein shown at the bottom was considerably necrotic. The xylem elements were necrosed, heavily stained with the safranin, and broken in numerous places as though brittle when cut. The thickenings in the walls could be discerned only with difficulty. The border parenchyma cells did not appear to be necrosed, but they contained nuclei that showed evidence of better fixation than in similar cells from normal-

appearing areas of the same leaf. These cells had apparently been more permeable to the fixative than normal. The mesophyll adjacent to this necrotic veinlet displayed some plastid disintegration, but did not stain like necrotic tissue. The xylem of the veinlet appeared to be the only portion of the leaf in this region that was completely necrotic.

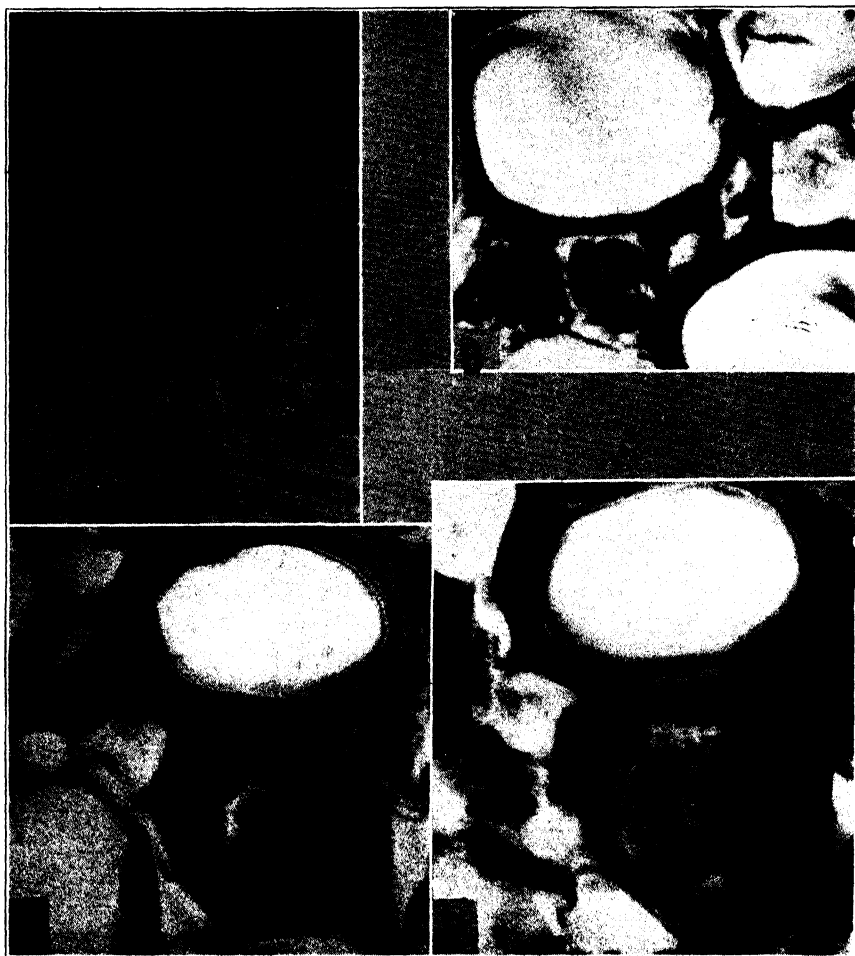


FIGURE 3. Primary lesions of ring-spot in Black-eye cowpea. A, tangential section of part of a lesion three days after inoculation showing necrosed xylem in a veinlet, $\times 360$; B, non-necrotic portion of a secondary vein in a lesioned area five days after inoculation, $\times 1300$; C, necrotic portion of the secondary vein shown in B. The tracheid at the bottom contains granular debris and tyloses, $\times 1260$; D, another section of the tracheid shown in C. The nucleus of one of the border parenchyma cells has been extruded into the tracheid, $\times 1300$.

In larger veins, containing phloem, the necrosis was often limited specifically to the xylem elements. In one case a vein was observed in which only one of the three xylem elements present was necrotic. This was the central element, and stood out in marked contrast to the normal-appearing elements on either side.

In one primary lesion, collected five days after inoculation, the xylem elements of a secondary vein that was involved were stained more heavily than normal with safranin. The tracheids sometimes contained, in addition to much granular debris, structures that appeared to be tyloses. In some cases these were small and bladder-like, extending from the wall into the lumen. Two such tyloses are shown in the large tracheid in Figure 3 C. A great deal of granular debris was also present. In many instances, however, the whole lumen had apparently been completely or almost filled by one or more tyloses. Most of the tyloses were filled with a dense undifferentiated mass that stained very heavily. Some were more lightly stained being less densely filled with the undifferentiated material.

In some of the tracheids structures that appeared to be large tyloses contained granular debris similar to that occurring in cells containing disintegrated plastids. In one instance the nucleus of the border parenchyma cell was present in the tracheid. While the nucleus was stained more heavily than normal with the safranin it was not disintegrated. It appeared in this case, that practically the whole parenchyma cell had been extruded into the tracheid lumen. This is illustrated in Figure 3 D which is a photomicrograph of another section of the debris-filled tracheid shown in Figure 3 C. The parenchyma cells bordering the tracheids that contained tyloses and granular debris were in various stages of necrosis and collapse.

In a non-necrotic portion of this same vein the tracheids were free of granular debris and tyloses. The bordering parenchyma cells were not collapsed, and contained normal-appearing plastids and nuclei. The tracheids were mature, being devoid of protoplasts and possessing fully thickened walls. Several tracheids and border parenchyma cells from this region are shown in Figure 3 B. In the severely necrosed area the granular debris, tyloses, etc., in the tracheids must have come from the adjoining parenchyma cells as in the less severely necrosed portions of the vein the tracheid lumina were entirely free of these materials.

The extension of the parenchyma cells into tracheid lumina had apparently occurred through the tracheid walls at points located in between the thickenings. Some of the parenchyma cells had apparently been ruptured, disgoring their contents into the lumina of the tracheids. The walls of the tracheids must have been greatly weakened or partly broken down either directly or indirectly by action of the virus. Intracellular bodies in this suspect were not observed in any of the lesions studied.

SUMMARY AND CONCLUSIONS

1. Lesions formed on Turkish tobacco in the summer, in the greenhouse, were typically much less necrotic than those formed in winter.

2. From studies of "summer" lesions in living Turkish tobacco leaves, it was found that the first visible signs of cellular breakdown were certain disintegrative changes in the chloroplasts and cytoplasm accompanied by a loss of water from the protoplast. The palisade parenchyma was generally affected first.

3. In primary "summer" lesions in Turkish tobacco there seemed to be a connection between necrotic zonation and alternating periods of light and darkness. The number of necrotic rings formed was not necessarily equivalent to the number of periods of darkness, although in the first few days of lesional development there was sometimes a rough approximation.

4. The cytological and especially the histological changes that occurred in the leaf as a result of virus action were conditioned to a considerable extent by the age of the tissues invaded.

5. The cytological modifications that occurred in both primary and systemic lesions in Turkish tobacco were essentially the same under ordinary greenhouse conditions in relatively mature leaves. The usual sequence of events in cell breakdown, as determined from sectioned and stained material appeared to be as follows: (a) modification of cytoplasm resulting in increased permeability of the protoplast; (b) disintegration of the chloroplasts either before, or at the time of (a); (c) nuclear degeneration following more or less complete disintegration of the chloroplasts and peripheral cytoplasm. Sometimes, however, little or no plastid disintegration occurred before death of the cell; (d) collapse of diseased cells due to desiccation of the affected tissue followed usually by oxidation to a brown color; (e) possible retardation of the development of plastids in chlorotic areas in systemically infected leaves.

6. In primary and systemic lesions formed on nearly mature leaves of Turkish tobacco little change in histological structure of the leaf occurred except the collapse and drying out of certain areas killed by the virus. In systemic lesions formed on young leaves, however, certain marked changes in histological structure were sometimes observed. These consisted of: (a) the rather specific action of the virus on the palisade parenchyma or on particular layers of the spongy parenchyma; (b) activation of the epidermis over completely necrotic areas of mesophyll; (c) the enlargement of living, more or less isolated, cells in certain partly necrotic areas of the mesophyll; (d) lack of differentiation of the mesophyll adjacent to necrotic areas.

7. The vascular tissues in Turkish tobacco leaves were more resistant to the action of the virus than the mesophyll.

8. Studies in susceptibles other than Turkish tobacco were limited pri-

marily to lesions formed on relatively mature leaves. In *Nicotiana rustica*, *N. glutinosa*, *N. glauca*, and *Petunia* sp. the cytological and histological pictures were much the same as those in nearly mature leaves of Turkish tobacco. In *Vigna sinensis* var. Black-eye, however, the vascular tissues of the leaf were much more susceptible to the virus than in Turkish tobacco. In this suscep the xylem elements of the veins were often necrosed before any other cells. In one lesion some of the tracheids of a necrotic secondary vein contained granular debris and tyloses.

9. The environmental conditions under which the plants were grown exerted a marked effect on the development of ring-spot lesions. Lesions formed in continuous darkness in water-saturated atmosphere were at first chlorotic, later becoming completely necrotic. The cells in the chlorotic area contained degenerate nuclei and granular debris, resulting from nearly complete plastid dissolution. In lesions formed in dry air in continuous darkness plastid breakdown was almost complete, but the affected cells collapsed and dried out rapidly. Hyaline spheres, about the size of chloroplasts, were observed in some diseased cells in primary lesions formed in continuous darkness. These may be similar to structures described by Sorokin (11) in mosaic-diseased tomato cells.

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DORMANCY IN TILIA SEEDS

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INTRODUCTION

Tilia fruits may remain in the ground several years and never produce a good stand of seedlings. No germination of fruits of *Tilia europaea parvifolia* resulted after four and one-third years, according to Puchner (8). He obtained 1 per cent germination of fruits which had had a small sector cut from the coat after five years. When the fruit coats were removed entirely and the seeds planted in a seed bed in January, the results showed 1 per cent germination after one and one-fourth years; 2 per cent after three and three-fourths years; 8 per cent after four and one-third years; and 14 per cent after six and three-fourths years with all remaining seeds rotten at that time. Chittenden (1) stated that basswood seed planted for four years remained dormant and viable. He described the difficulty as being due both to the impervious seed coat and the rudimentary embryo.

Workers have differed as to the effect of fall planting on the fruits of *Tilia*. Pammel and King in 1918 (7) reported abundant germination of *Tilia americana* by the middle of May when seeds were planted in the fall and stratified in a cold frame from which they were removed to the greenhouse in March. They obtained no germination in the greenhouse. On the other hand a progress report on the study of forest tree seed from Michigan State College Experiment Station in 1926 (6) showed no germination either in the greenhouse or in the nursery after an October 16th planting. Many other attempts to hasten the germination of these seeds including removal of the testa, and hot water and chemical treatments were unsuccessful. Chittenden (2) listed basswood as one of the tree seeds which should be planted in the fall since they germinated slowly and sometimes held over for a year if planted in the spring. He reported 2 per cent germination. Cobb (3, p. 127) summarized the basswood seed problem thus, "In the fall the hard nutlets hanging by a stem from a wing are not mature. The germ must have a year at least on the ground among the leaves and damp mold to ripen. In planting them it is, therefore, necessary to wait until the second spring for their germination." Tozawa (11) obtained seedlings of *Tilia amurensis* only by outdoor burial of the seeds before November. The outdoor temperature was as low as -3.5°C .

The effect of the seed coat and of different temperatures on germination has been studied by Rose (9), Grimsley (5), and Spaeth (10) with varying results. Rose (9) showed that the seed coats of *Tilia* interfered with water absorption to a considerable extent but he stated that water

absorption was not the only limiting factor to growth. He found that if the pericarps were removed and seeds placed on moist cotton at 4° to 6° C. for 78 days and then transferred to soil in the greenhouse they gave 71 per cent seedling production. If left at the low temperature for 90 days a still higher per cent of seedling production was obtained. Subsequent storage of seed at 0° to 2° C. failed to produce germination even after 140 days. This result he attributed to the low temperature since germination was obtained when the cultures were transferred to 10° to 12° C. For germination of *Tilia* he recommended a period of after-ripening in moist storage at 0° to 2° C., followed by two or three weeks at 10° to 12° C. until germination had begun, and finally a transfer to a still higher temperature to permit vigorous growth. He also suggested that one-year-old seeds were better than fresh seeds but stated that additional data upon this point were desirable. As after-ripening progressed, he found an increase in hydrogen ion concentration, as well as in the oxidase and catalase activities. Grimsley (5) thought that the seed coats of *Tilia* did not hinder the movement of water or gases to and from the embryo and therefore that dormancy existed in the embryo or in the endosperm or both. She further stated that seeds with testas removed or intact after-ripen and germinate at 5° C. A greater percentage of germination occurred at this temperature in seeds collected from northern trees than in seeds collected from southern trees. Freshly-harvested seeds after-ripened and germinated in less than five months when kept in moist storage at 5° C. for three months and then treated with weak HCl before planting. The strength of HCl and the time of treatment are not given. It was found that "there is greater catalase activity in moist seeds stored at 5° C. than in seeds stratified at 5° C." The method of "storage" or "stratification" is not described. Dry seeds stored at room temperature were also found to contain a large percentage of catalase. More recently Spaeth (10, p. 928) has described a successful method for hastening the germination of seeds of *Tilia americana*, involving the following steps: "Soften the pericarp by partial digestion in concentrated nitric acid; macerate on a screen in running water; dry and separate seed from pericarp remains by gravity; render testas water permeable by treatment with concentrated sulphuric acid; wash and place in refrigeration in a moist sterile medium providing good aeration." The last step required three to five months at a temperature just above freezing.

The present paper deals mainly with dormancy in the seeds of *Tilia americana* L. but experiments on three other species are also reported.

METHODS AND MATERIALS

Cultures in constant temperature ovens consisted of a mixture of moist granulated peat moss and the fruits or seeds to be tested. If the germination under any specific temperature condition was to be determined the

culture was left in that condition until germination had occurred or until the termination of the test. In most cases, however, the object was to determine the conditions necessary for after-ripening, or preparation for seedling production in the greenhouse. For this purpose low constant temperatures (1° , 5° , and 10° C.) and higher constant temperatures (15° , 20° , or 25° C.) followed by low were used for treatment. Samples of fruits or seeds were removed from the oven peat cultures at monthly intervals. These samples were then planted in greenhouse flats in a mixture of composted sod soil, sand, and granulated peat moss. Seedling production was then noted. When daily alternating temperatures were used, the cultures were kept at the higher temperature 8 hours and at the lower temperature 16 hours each day.

The peat cultures were examined once each week, when needed water was added, and a germination count made. In most cases these seedlings were discarded.

Many fruits or seeds were planted directly in flats in the mixture described above and the flats were then placed in cold frames or in the greenhouse. Three cold frame treatments were used. In the open frame the seeds were exposed to all climatic changes including freezing and thawing. Frames covered with boards afforded protection but permitted a widely fluctuating temperature, while the application of a leaf mulch before the board cover was added provided a narrow temperature range.

Two methods were used to extract seeds from fruits. For the preliminary tests the seeds were removed by hand. Later the nitric acid-extraction method described by Spaeth (10) was used. This latter method proved ineffective in removing fruit coats even when the extraction was prolonged for as much as four hours. After this long nitric acid-extraction period the seeds seemed to be slightly injured as evidenced by their later germination. This injury may have been due to the subsequent necessary maceration however, rather than to the harmful effect of the acid treatment. In order to eliminate these doubtful factors, the majority of the tests were conducted with hand-extracted seeds. Extracted seeds were treated with concentrated sulphuric acid for 20 minutes (Spaeth 10) after which they were either mixed with moist granulated peat moss and put in the ovens or planted directly in flats. Some temperature effects were also determined with extracted seeds treated with sulphuric acid for 10, 30, and 60 minutes. For pre-treatment at various temperatures enough seeds were used to permit greenhouse sample plantings of 100 each over a period of several months. Five hundred fruits and one or two hundred seeds each were used in the outside plantings.

The amount of water absorbed by fruits and seeds intact and with coats removed was determined at room temperature in open bottles. The outsides of fruits or seeds were carefully dried with filter paper and placed

in air-tight bottles before each weighing. The experiment was continued until a constant weight was obtained in each case.

Embryos were excised from seeds which had been treated under various conditions previously or had been soaked overnight at room temperature after the coats had been removed. These embryos were then placed on moist filter paper at room temperature.

The method used for the determination of catalase activity was essentially the same as that described by Davis (4) except that calcium carbonate was used to neutralize the hydrogen peroxide. Twenty-five seeds were used for each test. When dry fruits or fruits from various temperatures were tested the fruit coats were always removed just before the catalase tests were made. The seeds were ground with a small amount of sand and calcium carbonate and then distilled water was added to bring the volume up to 50 cc. To 5 cc. aliquots of this solution, 10 cc. of distilled water were added for each test.

All of the seed material used was obtained from Thomas J. Lane, Dresher, Pennsylvania. *Tilia americana* L. was collected in the United States while *T. tomentosa* Moench., *T. platyphyllos* Scop., and *T. cordata* Mill. were imported from Austria.

RESULTS AND DISCUSSION

TILIA AMERICANA

Oven Tests and Greenhouse Sample Plantings

Test 1. Preliminary tests were begun in 1929 using seeds harvested in the fall of that year. The first tests were made using both intact fruits and hand-extracted seeds. These were put in granulated peat moss in bottles which were placed at constant temperatures of 15°, 20°, 25°, and 30° C. and at daily alternating temperatures of 10° to 20° C., 10° to 30° C., and 15° to 30° C. The total number of seedlings produced in these tests was eight and these appeared at 15° C., 10° to 30° C., and 15° to 30° C. These results pointed to the need of pre-treatment for germination.

Fruits and seeds mixed with moist granulated peat moss at 1°, 5°, and 10° C. produced a few seedlings at the low temperatures, especially 5° and 10° C. after three months to a year. Sample plantings of fruits and seeds made in the greenhouse after one, eight, and eleven months at 1°, 5°, or 10° C. produced no seedlings. The samples planted after one month at low temperatures were left in the greenhouse until February 1931 when the flat was placed in an open cold frame. In April 1931 the fruit sample had germinated to the extent of 24 per cent while the seeds had given 38 per cent seedling production.

Test 2. Additional experiments were begun in February 1931 with the same seed lot used in test 1. These seeds were, therefore, two years old when new tests were started. The delay in germination was thought to be

due to coat or endosperm characters since the embryo was quite large and apparently ready to grow. It then seemed possible that a high temperature preceding the low temperatures used for pre-treatment might soften the seed coat and hasten germination. Twenty-five degrees C. was arbitrarily selected as the high temperature to be used. Both fruits and hand-extracted seeds were again used. Mixtures of the material to be tested and moist granulated peat moss were placed at 25° C. for one month after which they were transferred to 1°, 5°, 10°, and 15° C. The greatest production of seedlings obtained in the greenhouse from sample plantings of fruits thus treated was 2 per cent which followed six months at 5° C. preceded by one month at 25° C. Other seedlings produced consisted of 1 per cent after four, five, six, and seven months at 1° C. and seven months at 5° C.

Seeds gave slightly better seedling production than fruits. Five and 3 per cent were obtained in the greenhouse after three months at 1° and 5° C., respectively. No sample plantings were possible after four months at low temperatures as all the seeds had rotted by that time.

Obviously the clue to the difficulty in germinating *Tilia* had not yet been found, so additional experiments were planned.

Test 3. The probable beneficial effect of a longer period at high temperature preceding low temperature treatment and the need for extending the range of the high temperatures used, formed the bases for a new series of tests. Again the 1929 seed crop was used and experiments were started in June 1931. A limited supply of seeds of *T. americana* permitted the use of 25° C. alone as the high temperature for one, two, and three months followed by 1°, 5°, or 10° C. for one to eight months. Few seedlings were produced.

Test 4. A new lot of seeds was obtained in the fall of 1931. Test 3 was repeated using 15°, 20°, 25°, and 30° C. for one, two, three, four, and five months preceding the low temperature treatment at 1°, 5°, or 10° C. Better seedling production resulted than in any of the previous oven tests (Table I). The best high temperature tried was 20° C. Twelve and 14 per cent seedling production was obtained in the greenhouse after four months at 20° C. followed by five months at 1° and 5° C.

Test 5. The efficacy of the initial high temperature was thought to be due to a seed coat effect. Consequently, experiments with seeds of the 1932 crop were planned to study the rôle of the seed coat in delayed germination of *T. americana*. A cutting test of 3400 fruits of this crop showed an average of 95.5 per cent apparently good seeds present. A cutting test of 500 of these seeds revealed 14 per cent empty or rotten in spite of their good appearance. The nitric acid-extraction method and the sulphuric acid treatment of the extracted seeds reported here are essentially those used by Spaeth (10). Tests at constant low temperatures and at 20° C. for

TABLE I
SEEDLING PRODUCTION IN THE GREENHOUSE FROM FRUITS OF FOUR SPECIES OF TILIA AFTER PRE-TREATMENT AT VARIOUS TEMPERATURES FOR DIFFERENT PERIODS

[illegible]

TABLE I (Continued)

Pre-treatment			T. platyphyllos					T. cordata					
High temp.	Months	Low temp. °C.	Per cent seedling production after months at low temp.					Per cent seedling production after months at low temp.					
			1	3	5	7	9	1	3	5	7	9	
None		1 5 10	0	0	0	0	4	—	1	4	1	6	
			0	0	0	0	2	0	—	0	1	1	0
			0	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	2	0	0	4	4	2	2
			0	0	0	0	0	1	0	1	6	—	—
15	1	1 5 10	0	0	2	0	0	0	0	0	0	0	
			—	0	2	2	0	6	13	8	2	2	
			—	0	0	2	0	7	1	8	—	0	
			0	2	14	2	—	0	11	15	10	—	
			0	0	4	2	—	0	2	16	17	—	
15	3	1 5 10	0	2	0	0	0	1	0	0	1	—	
			0	0	0	4	2	—	1	0	0	—	
			0	0	0	0	0	0	0	0	0	—	
			0	4	6	2	—	0	24	30	16	—	
			0	2	4	0	—	1	20	17	16	—	
15	4	1 5 10	0	0	0	0	0	—	0	0	2	5	
			0	4	6	2	—	0	24	30	16	—	
			0	2	4	0	—	1	20	17	16	—	
			0	0	2	0	—	0	0	2	5	—	
			0	0	0	0	—	0	0	0	0	—	
15	5	1 5 10	0	8	22	—	—	0	10	46	—	—	
			0	4	6	—	—	0	7	18	—	—	
			0	2	0	—	—	0	0	1	—	—	
			0	0	0	0	—	0	0	0	0	—	
			0	0	0	0	—	0	0	0	0	—	
20	1	1 5 10	0	0	0	0	0	0	6	4	1	9	
			0	0	0	0	0	0	2	3	3	0	
			0	0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	0	
20	2	1 5 10	—	8	2	4	12	1	19	17	10	19	
			—	2	0	4	0	3	15	9	5	3	
			—	0	6	2	4	0	0	0	0	0	
			0	4	16	20	—	0	24	28	12	—	
			0	0	2	14	—	1	3	15	—	—	
20	3	1 5 10	0	0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	0	
20	4	1 5 10	0	22	8	10	—	0	41	40	—	—	
			0	28	8	10	—	0	17	27	—	—	
			0	2	0	8	—	0	0	2	10	—	
			0	0	0	0	—	0	0	0	0	—	
			0	0	0	0	—	0	0	0	0	—	
20	5	1 5 10	2	26	36	—	—	0	12	—	—	—	
			0	12	8	—	—	0	33	—	—	—	
			0	0	0	4	—	0	0	0	0	—	
			0	0	0	0	—	0	0	0	0	—	
			0	0	0	0	—	0	0	0	0	—	

various periods followed by low temperatures were again tried using fruits, hand-extracted seeds, hand-extracted seeds treated with sulphuric acid, nitric acid-extracted seeds, and nitric acid-extracted seeds treated with sulphuric acid. The usual sulphuric acid treatment was for 20 minutes but 10, 30, and 60 minutes were also used. Ten minutes proved less effective than any of the other treatment periods. Thirty and 60 minutes appeared as good as 20 minutes for the experiments tried.

The general appearance of the cultures of these variously treated seeds after about two weeks in the ovens was as follows: fruits and hand-extracted seeds in good condition; nitric acid-extracted seeds slightly moldy; sulphuric acid-treated seeds quite moldy. These conditions obtained at both high and low temperatures.

Again fruits and untreated extracted seeds produced very few seedlings in the greenhouse even after a prolonged length of time in the ovens (Table

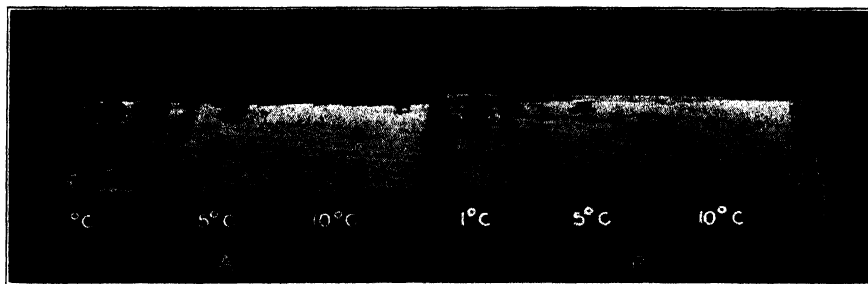


FIGURE 1. *Tilia americana*. Seedling production from extracted seeds treated with concentrated sulphuric acid for 20 minutes, then pre-treated for three months at 1°, 5°, and 10° C. Photographed 15 days after planting in the greenhouse. (A) Hand-extracted; (B) nitric acid-extracted.

II). However, seeds extracted from their fruit coats and treated with concentrated sulphuric acid for 20 minutes, after-ripened in three (Fig. 1) or four months at 1° C. giving 64 and 68 per cent seedling production in the greenhouse when sample plantings were made. Five degrees C. was inferior to 1° C., and 10° C. was apparently too high (Fig. 1). Many of these treated seeds rotted in a very short time at 20° C. so the transfers to lower temperatures were limited. Any good seeds which were transferred from 20° C. to 5° C. received no advantage from the period at high temperature. These oven and sample planting tests were repeated with similar results.

Many seedlings appearing in the low temperature ovens grew well when planted in greenhouse pots. However, a few seedlings thus planted never appeared above ground. The number growing and the rate of growth was about the same regardless of the previous treatment of the seed. The sul-

TABLE II

PRODUCTION OF *TILIA AMERICANA* SEEDLINGS IN THE GREENHOUSE FOLLOWING
PRE-TREATMENT AT VARIOUS TEMPERATURES FOR DIFFERENT PERIODS

Material	Pre-treatment			Per cent seedling production after months at low temp.							
	High temp.		Low temp. ° C.	1	2	3	4	5	6		
	° C.	Months									
Hand-extracted seeds	None		1 5 10		0 0 0	0 1 0	0 0 0	0 0 0	0 0 0		
		20	1 2 3 4	5	0 0 0 0	0 0 1 0	0 0 0	0			
		Hand-extracted H ₂ SO ₄ -treated seeds	None		1 5 10		6 13 2	57 18 4	64 25 3	0 0 4	
				20	1 2 3 4	5	0 0 0 0	0 0 0 1	0 0 2	16	
HNO ₃ -extracted seeds	None				1 5 10		0 0 0	0 0 0	1 0	1 0	0 0
				20	1 2 3 4	5	0 0 0 0	0 0 0	0 0	0	
		HNO ₃ -extracted H ₂ SO ₄ -treated seeds	None		1 5 10		2 11 2	42 13 1	68 13		
				20	1 2 3 4	5	0 0 0 0	0 0 0	4 0 0	15 14	
Fruits	None				1 5 10		0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
				20	1 2 3 4	5	0 0 0 0	0 0 0 1	1 0	0	

phuric acid treatment hastened the initiation of growth but subsequent growth up to five months apparently was not affected.

Cultures were also left at 20° C. throughout the course of the experiment. Very few germinations occurred even in the cases of the sulphuric acid-treated seeds. This pointed to a further dormancy inside the seed coat.

Embryos of seeds after-ripened at low temperatures and non-after-ripened seeds were then excised to determine whether this dormancy lay in the embryo. Since the embryo is very large and the cotyledons are entangled in the endosperm, many of them were injured when they were excised. That the embryo is at least partially dormant was shown when embryos were excised from hand-extracted seeds, not treated and treated with sulphuric acid, nitric acid-extracted seeds, not treated and treated with sulphuric acid, and fruits which had been in moist granulated peat moss for two months at 1°, 5°, and 10° C. and for one month at 20° C. followed by one month at 5° C. Hand-extracted seeds from fruits stored dry in the laboratory were used as controls. In the latter case the seeds were soaked in water overnight at room temperature in order to make possible the removal of the embryo. All excised embryos were placed on moist filter paper in diffuse daylight at room temperature. The average initial



FIGURE 2. *Tilia americana*. Growth of excised embryos after 5 days on moist filter paper. (A) Control (fruits stored dry in laboratory); (B) extracted seeds kept one month at 20° C. followed by two months at 5° C.; (C) extracted seeds treated with concentrated sulphuric acid for 20 minutes and then kept one month at 20° C. followed by two months at 5° C.

length of the hypocotyl of embryos at the time they were excised was approximately 0.4 cm. After three days at room temperature embryos from sulphuric acid-treated seeds were larger than the others and had greener cotyledons and pink healthy looking roots with an abundance of root hairs. This may be seen in Figure 2. These embryos had increased to from 1 to 1.3 cm. in three days while others had grown very little, measuring 0.5 to 0.6 cm. in length. All of the embryos, however, including the control, showed some evidence of growth. The cotyledons always turned green and a few root hairs appeared.

All excised embryos still in good condition after four days were transferred to soil in individual three-inch pots which were placed in the greenhouse. About 50 per cent of these developed subsequently regardless of previous treatment and produced, for the most part, plants of normal appearance although there was indication of stunting in a few cases. In all

cases of stunting the cotyledons had been injured in the process of excising. However, many injured embryos developed normally.

Embryos were also removed after three months of pre-treatment with similar results.

The results of water absorption tests expressed in per cent of the original weight of the material were as follows: naked seeds, 97.8 in 1 day; hand-extracted seeds with coats intact, 21.8 in 6 days; hand-extracted seeds treated with concentrated sulphuric acid for 20 minutes, 83.6 in 7 days; nitric acid-extracted seeds, 11.8 in 7 days; nitric acid-extracted seeds treated with concentrated sulphuric acid for 20 minutes, 94.9 in 7 days; entire fruits, 95.3 in 14 days. In case of the fruit, however, most of the water was held in the fruit coat and in the space between the fruit coat and the seed. Although the naked seeds and seeds treated with sulphuric acid absorbed the greatest amount of water, a considerable amount was also absorbed by hand-extracted or nitric acid-extracted seeds with the coats intact. Absorption of water, then, may be a limiting factor in germination but the seed coat probably also limits the gaseous exchange.

The relation of catalase activity to the degree of after-ripening of the seed was also studied. Tests were made of fruits, hand-extracted seeds, and hand-extracted seeds treated for 20 minutes with concentrated sulphuric acid after one, two, and four months at 1°, 5°, 10°, and 20° C. Seeds from 5° and 20° C. were tested after three months and as many seeds as were still available were tested after five months. Catalase activity was also determined after one month at 20° C. followed by one, two, three, and four months at 5° C.; two months at 20° C. followed by one, two, and three months at 5° C.; and three months at 20° C. followed by one and two months at 5° C.

The results of these tests are shown in Table III. It will be seen that the catalase activity of the fruits remained about the same regardless of the temperature or duration of pre-treatment. This activity is also very similar to that of the dry control lots of seeds.

Although the hand-extracted seeds showed a slight increase in catalase activity after two months at low temperatures, no additional increase was shown up to five months and the entire record for these seeds exceeded by very little the record for fruits.

On the other hand, seeds treated with sulphuric acid showed considerable increase in catalase activity after one month at all temperatures except 1° C. There was a great increase at all temperatures after two months with very little additional increase for longer periods. The high catalase activity for 20° C., in spite of the fact that a very small percentage of seedlings ever appear at that temperature without previous low temperature treatment, indicates that catalase activity alone cannot be used as a criterion for determining the completeness of after-ripening. The same

TABLE III

CATALASE ACTIVITY OF SEEDS OF *TILIA AMERICANA* AFTER PRE-TREATMENT
AT VARIOUS TEMPERATURES FOR DIFFERENT PERIODS

Seed material	Pre-treatment		Cc. O ₂ released after minutes							
	Temp. ° C.	Months	0.5	1.0	1.5	2.0	3.0	4.0	5.0	
Control (dry fruits)			0.4	1.6	2.6	3.7	5.4	6.8	8.1	
			0.9	2.1	4.1	5.9	9.5	12.7	15.5	
			0.8	1.9	3.4	4.7	7.1	9.1	10.9	
			0.6	1.9	3.1	4.4	7.6	9.5	11.5	
			1.0	2.3	3.7	4.9	7.1	9.1	10.7	
Hand-extracted seeds	1	1	0.7	1.9	2.9	3.9	5.5	6.8	7.8	
		2	1.2	3.0	5.3	7.4	11.6	15.0	17.7	
		4	0.9	2.7	4.5	6.3	9.0	11.5	13.6	
		5	0.9	2.4	4.4	6.1	9.3	11.6	13.7	
	5	1	0.9	2.1	3.3	4.3	6.1	7.7	8.8	
		2	0.8	2.9	5.1	7.4	11.5	14.9	17.7	
		3	1.6	4.0	6.3	8.8	12.4	15.0	17.2	
		4	1.3	2.9	4.7	6.6	9.9	12.3	14.0	
		5	0.8	2.1	3.5	4.7	7.1	9.2	10.8	
	10	1	0.1	1.2	2.4	3.5	5.5	7.3	8.7	
		2	0.9	2.5	4.4	6.2	9.8	12.6	15.0	
		4	0.8	2.2	3.6	5.0	7.3	9.0	10.6	
		5	0.5	2.0	3.7	5.0	7.5	9.4	11.1	
	20-5	1+1	0.5	2.1	3.7	5.2	8.3	10.9	13.2	
		1+2	1.0	2.2	3.7	5.4	8.2	10.8	12.9	
		1+3	1.5	4.1	6.4	9.0	13.3	16.7	19.3	
		1+4	0.6	2.0	3.6	5.4	8.7	10.6	12.2	
	20-5	2+1	1.5	3.6	5.4	6.9	9.5	11.6	13.3	
		2+2	1.7	3.7	5.9	7.7	10.9	13.7	15.8	
		2+3	1.2	2.6	4.3	5.8	8.4	10.9	13.1	
	20-5	3+1	1.0	2.1	3.9	4.7	7.6	9.6	11.4	
		3+2	1.0	2.5	3.9	5.8	8.8	11.8	14.1	
	20-5	4+1	1.2	3.5	5.6	7.5	10.5	12.9	14.9	
	20	1	0.0	0.6	1.7	2.1	4.6	6.1	7.3	
		2	1.0	3.0	5.3	7.4	10.7	13.3	15.5	
		3	0.7	1.9	3.2	4.5	7.0	9.1	10.9	
		4	1.4	3.4	5.7	7.7	11.4	14.3	16.5	
Hand-extracted seeds, treated 20 min. with conc. H ₂ SO ₄	1	1	1.2	2.7	4.4	5.9	8.2	10.3	12.3	
		2	5.3	12.5	19.3	25.3	35.4	39.8		
		4	9.5	18.7	26.5	33.4				
		5	10.7	21.9	30.7	38.3				
	5	1	2.4	5.8	8.8	11.3	16.2	19.9	22.7	
		2	11.6	25.6	37.4					
		3	12.7	24.9	35.2					
		4	8.5	18.3	27.3	35.3				
	10	1	3.8	9.1	13.5	18.0	26.4	33.0	38.0	
		2	13.1	26.6	38.3					
		4	5.4	10.6	17.6	23.2	32.1	38.7		

TABLE III (Continued)

Seed material	Pre-treatment		Cc. O ₂ released after minutes						
	Temp. °C.	Months	0.5	1.0	1.5	2.0	3.0	4.0	5.0
(Cont.) Hand-extracted seeds, treated 20 min. with conc. H ₂ SO ₄	20-5	1+1	11.5	25.5	38.3				
		1+2	9.3	19.9	29.0	36.2			
		1+3	8.0	19.5	28.8	36.8			
		1+4	6.4	15.1	23.1	30.2	39.9		
	20-5	2+1	5.4	13.3	21.2	27.8	38.7		
		2+2	4.7	11.3	17.7	23.3	32.7		
		2+3	4.5	11.1	17.0	22.9	31.9	38.4	
	20-5	3+1	3.0	8.1	13.6	18.9	27.9	36.0	
	20	1	2.0	5.5	9.1	12.3	18.3	22.4	25.8
		2	4.5	13.0	21.4	28.6			
		3	3.3	8.1	13.5	18.0	25.9	32.0	37.3
Fruits	1	1	1.3	2.7	4.1	5.6	8.1	10.1	11.7
		2	1.2	2.9	5.0	7.0	11.0	14.6	16.9
		4	1.2	3.3	4.3	5.8	8.0	10.6	12.7
		5	0.6	1.8	3.2	4.7	6.9	9.3	11.0
	5	1	0.1	1.1	2.3	3.5	5.8	7.6	8.8
		2	0.8	2.6	4.8	6.9	10.7	13.5	16.2
		3	1.2	2.6	4.2	5.8	8.9	11.2	13.2
		4	1.4	2.9	4.2	5.7	8.8	11.1	13.9
		5	0.0	1.1	2.6	4.1	6.9	9.1	11.1
	10	1	0.9	2.7	4.6	6.1	9.0	11.4	13.2
		2	1.2	2.6	4.6	6.5	10.5	14.2	17.0
		4	1.3	2.9	4.1	5.7	8.2	10.7	13.4
		5	0.8	2.2	4.2	5.5	8.0	10.0	11.8
	20-5	1+1	1.0	3.8	6.7	9.1	13.7	17.2	19.8
		1+2	1.4	3.0	5.0	6.9	10.3	13.2	15.5
		1+3	1.3	3.0	4.9	6.7	9.7	12.6	14.8
		1+4	0.5	2.3	4.1	5.7	8.7	10.9	12.9
	20-5	2+1	1.3	3.2	5.2	6.9	10.1	12.2	14.3
		2+2	1.4	3.0	4.7	6.3	8.9	11.2	13.6
		2+3	1.0	2.8	4.7	6.4	10.3	13.2	14.5
	20-5	3+1	1.0	3.0	5.3	7.1	10.3	13.2	15.8
		3+2	1.3	3.0	4.9	6.2	8.6	11.0	13.3
	20-5	4+1	1.3	3.0	4.6	6.3	9.7	11.7	14.0
	20	1	0.3	1.5	2.9	4.1	6.5	8.6	9.6
		2	1.0	2.7	4.6	6.7	10.4	13.7	16.6
		3	1.2	2.6	4.5	6.0	8.6	10.7	12.5
		4	1.2	3.1	5.1	7.2	10.1	12.3	14.5

might be said of the high catalase activity in the case of high temperature followed by low, which condition is quite ineffective in bringing about seedling production in the greenhouse. Increased activity of this enzyme undoubtedly accompanies the after-ripening process but some other factor or set of factors is involved. This fact is also shown by the constancy of the

enzyme action after two months at various low temperatures; yet better seedling production was obtained after three or four months (Table II).

Flat Plantings

Test 1. Preliminary tests with fruits and hand-extracted seeds were begun in November 1929. Plantings were made in flats which were placed

TABLE IV
PER CENT SEEDLING PRODUCTION FROM FRUITS OF FOUR SPECIES OF *TILIA*
PLANTED OUTSIDE ON DIFFERENT DATES

Winter condition	Month planted	<i>T. americana</i>			<i>T. tomentosa</i>		
		After 1 winter		After 2 winters	After 1 winter		After 2 winters
		1929 crop*	1931 crop	1929 crop*	1929 crop*	1931 crop	1929 crop*
Open cold frame	Oct.	2	0	4	0	0	2
	Sept.	4		11	0		5
	Aug.	8	2	12	0	2	5
	July	15	2	17	1	2	6
	June	25	6	32	2	5	8
Mulched cold frame	Oct.	1	2	4	0	2	2
	Sept.	1		8	1		9
	Aug.	13	4	20	0	2	8
	July	7	8	9	2	3	9
	June	36	7	39	2	2	5
Board-covered cold frame	Oct.	4	0	18	1	2	4
	Sept.	6		13	1		6
	Aug.	19	7	26	2	4	10
	July	13	3	22	2	4	12
	June	22	9	30	2	12	14
Greenhouse		0		0	0		
		<i>T. platyphyllos</i>			<i>T. cordata</i>		
Open cold frame	Oct.	6	1	8	7	3	10
	Sept.	12		18	12		17
	Aug.	9	7	12	13	25	22
	July	10	17	11	17	24	24
	June	22	15	23	24	22	18
Mulched cold frame	Oct.	24	1	27	15	5	16
	Sept.	52		41	16		21
	Aug.	37	10	42	15	0	22
	July	1	4	1	20	4	26
	June	34	28	34	28	8	36
Board-covered cold frame	Oct.	18	4	22	8	14	7
	Sept.	25		43	11		17
	Aug.	50	25	31	27	30	44
	July	44	34	51	28	28	40
	June	38	46	42	24	34	33
Greenhouse		0			0		0

* Seeds 2 years old when plantings were made.

immediately in the greenhouse and in open, mulched, and board-covered cold frames. No seedlings were produced in the cold frames in 1930 and only up to 9 per cent in 1931. From those kept in the greenhouse only one seedling was produced in the two-year period.

Test 2. Since it was thought probable that a high temperature prior to the cold treatment might be beneficial, plantings of the same 1929 crop of fruits were made in June, July, August, September, and October 1931. If the high temperature proved effective a better stand of seedlings should be obtained from the June than from the September or October planting. This was found to be true as shown in Table IV and Figure 3. The seeds planted

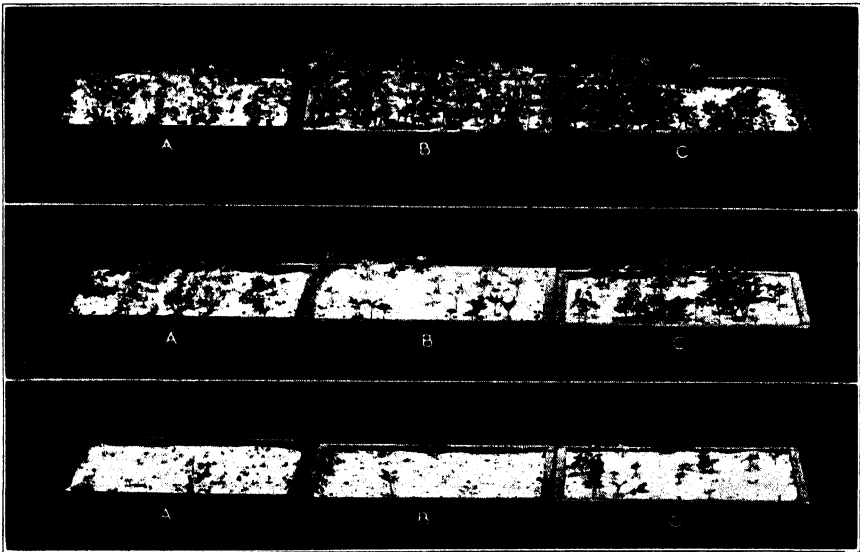


FIGURE 3. *Tilia americana*. Seedling production from fruits planted in flats and placed in cold frames. Kept during the winter in (A) open, (B) mulched, and (C) board-covered conditions. Photographed May 1932. Seeds were planted (1) in June, (2) in July, and (3) in September 1931.

in June had approximately four months of high temperature before the beginning of cold weather although September had some cool days. Similarly, seeds planted in July, August, September, and October had approximately three, two, one, and no months at high temperature preceding the low. It will be seen from Table IV that when seeds are planted in June, they give from 22 to 36 per cent seedling production the following spring. Those planted in July or August had somewhat smaller percentages while those planted in September and October gave very poor seedling production after the first winter. Total seedling production after the second winter outside is also shown in Table IV. In all cases, additional production of

plants was obtained after the second winter but the fall plantings still gave low percentages of seedling production.

The June, July, and September plantings are shown in Figure 3. These indicate beyond a doubt the benefit of summer planting. (A) shows the open frame condition where freezing and thawing occurred; (B) the mulched condition which fluctuated little in temperature but was always somewhat above freezing; and (C) the board-covered condition which had less temperature fluctuation than the open frame but permitted freezing and thawing. The earliest appearance of seedlings was from flats wintered in the board-covered condition while the latest to appear were from flats in the open frame (Fig. 3).

It will be noted that seedling production from these outside plantings was much greater than that obtained from oven tests of the same seed crop.

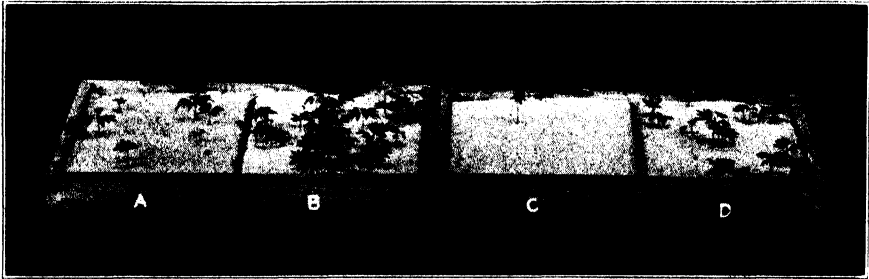


FIGURE 4. *Tilia americana*. Seedling production from extracted seeds planted in mulched frame January 1933. Photographed May 1933. (A) Hand-extracted; (B) hand-extracted treated with concentrated sulphuric acid for 20 minutes; (C) nitric acid-extracted; (D) nitric acid-extracted treated with concentrated sulphuric acid for 20 minutes.

Test 3. Plantings similar to those reported in test 2 were made using the seeds of the 1931 crop. Seeds were sown in flats in June, July, August, and October 1932. Results of these tests after one winter followed the general trend of the previous experiment although the seedling production here was much lower than previously (Table IV). In this case plantings were also made in January, February, and March 1932. These showed up to 8 per cent seedling production in the summer of 1933.

It is interesting to note that an initial period at low temperature, such as the seeds had when they were planted in the late fall, seemed actually to reduce the germination capacity since the percentage seedling production was small even after a second winter when the intervening months of high temperature had had a chance to bring about preliminary changes necessary for after-ripening. This same condition obtained for the January, February, and March plantings. The initial cold period may injure the

embryo by preparing it to grow before its seed coat will allow the entrance of the necessary water and oxygen. Most of the ungerminated fruits are rotten after the second winter.

That summer planting hastens germination by rendering the seed coat more permeable is again shown in Figure 4. Here the fruit coats were removed by using nitric acid or by hand, and samples of each were treated with concentrated sulphuric acid for 20 minutes. These were then planted in flats and placed in a mulched frame in January 1933. Seedling production indicated very clearly the advantage of sulphuric acid treatment. The photograph shows the appearance of the flats in May 1933, four months after planting. It will be noticed that the nitric acid-extracted seeds gave fewer seedlings than the hand-extracted. This was probably due to injury in extraction. (See Methods.)

The general results from plantings made directly in flats were in accord with the findings from sample plantings made in the greenhouse after various periods in constant temperature ovens. Except for sulphuric acid-treated seeds, however, germination percentages were higher in the case of flat plantings.

OTHER *TILIA* SPECIES

Oven Tests and Greenhouse Sample Plantings

Tests made on *T. americana* and reported under oven tests 1, 2, and 3 were repeated for seeds of *T. cordata*, *T. platyphyllos*, and *T. tomentosa*. A summary of the results will be given here.

Test 1. The preliminary tests showed *T. tomentosa* similar to *T. americana* in results obtained. Slightly better germination was noted for *T. platyphyllos* which gave up to 4 per cent seedling production from samples of fruits and seeds planted in the greenhouse after four months at 5° C. Similarly, *T. cordata* gave few seedlings from any of the conditions tried.

Test 2. T. platyphyllos. The best seedling production from fruits was 10 per cent obtained in the greenhouse after five months at 1° C. However, 7 per cent was obtained after four months at 1° or 5° C. or five months at 5° C. Hand-extracted seeds after-ripened a month or two sooner but showed no improvement over the fruits in the percentage of seedlings produced.

T. cordata. Contrary to the report of Puchner (8) this species proved easiest to germinate throughout the tests employed. In this series, for instance, the fruits gave 24 per cent germination in the greenhouse after seven months at 1° C., while seeds similarly planted produced seedlings to the extent of 22, 16, and 20 per cent after four months at 1°, 5°, and 10° C., respectively.

It will be noted that neither of these species benefited by the high temperature preceding the low. Although later tests showed the beneficial

effect of such treatment, the increase in germination was never so marked as for *T. americana*. *T. tomentosa* was not included in this test.

Test 3. T. tomentosa. Only occasional seedlings were obtained from sample plantings in soil after one to nine months at constant temperatures of 1°, 5°, or 10° C. or after one, two, or three months at 15°, 20°, or 25° C., followed by one to eight months at 1°, 5°, and 10° C.

T. platyphyllos. High temperature followed by low was effective for after-ripening in this case. The highest percentage obtained from sample plantings from constant low temperature treatment was 10 (after five months at 5° C.) whereas 28 per cent seedling production followed a treatment of three months at 20° C. preceding four months at 1° or 5° C. Comparable seedling production (24 per cent) resulted from a previous period at 15° C., followed by four months at 5° C. It should be noted that 20° C. was superior to 15° or 25° C. for high temperature treatment.

T. cordata. Results of these tests were essentially the same as those for *T. platyphyllos*.

Test 4. When test 3 was repeated using the high temperatures for longer periods of time (1, 2, 3, 4, and 5 months), before the cultures were transferred to low temperatures, all three species showed improvement over the previous oven-sample planting tests (Table I). The efficacy of a longer period at high temperature, however, was not so marked after three months. Prolonged high temperature treatment rotted many of the seeds, especially at 25° C. and 30° C., so that the supply of seeds to be transferred to low temperatures became very much reduced. Seeds of *T. cordata* were particularly susceptible to decay organisms. Germination of *T. tomentosa* remained very poor throughout the experiments.

Flat Plantings

T. tomentosa. In spite of the low seedling production, the beneficial effect of summer planting is also apparent here (Table IV), although it does not show definitely until after the second winter in the case of the 1929 crop. The 1931 crop, however, planted in June and kept in a board-covered frame over winter gave 12 per cent seedling production the following summer. This compares favorably with any percentage obtained at any time from these seeds. No sulphuric acid treatment has been tried but additional experiments are needed to determine a satisfactory method for germinating seeds of this species.

T. platyphyllos and *T. cordata.* Both of these forms germinated with comparative ease and with relatively high percentages throughout the tests. The need for low temperature treatment was evident, however, and the results of the flat plantings (Table IV) indicated in general the same beneficial effect of high temperature prior to low temperature as was evidenced by the more difficult forms of *T. americana* and *T. tomentosa*. That

the summer planting is not so important in the two former species as in the two latter is shown very clearly in a comparison of Figures 3 and 5, where summer and fall planting effects on *T. americana* and *T. platyphyllos* are shown. The low germination of the July planting of both 1929 and 1931 seed crops of *T. platyphyllos* is striking (Table IV). This may be due to excessively high temperatures for two months following planting. Oven tests showed that temperatures higher than 20° C. were unfavorable in all cases and this form may be more sensitive to heat. More data are needed on this point.

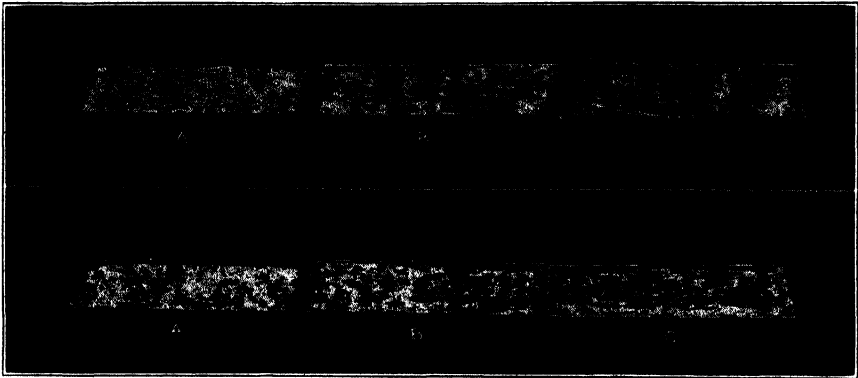


FIGURE 5. *Tilia platyphyllos*. Seedling production from fruits planted in flats and placed in cold frames. Kept during the winter in (A) open, (B) mulched, and (C) board-covered conditions. Photographed May 1932. Seeds were planted (1) in June, and (2) in September 1931.

CONCLUSIONS AND SUMMARY

Dormancy in seeds of *Tilia americana* is due to an impermeable seed coat and a partially dormant embryo. To bring about germination, then, two steps are necessary. The seed coats must be rendered permeable and the embryo must be after-ripened.

The first step was accomplished by two methods. The first of these consisted in placing the fruits or extracted seeds in moist granulated peat moss or in soil at a temperature of about 20° C. for four months. A lower temperature (15° C.) was less effective and higher temperatures (25° or 30° C.) often caused decay. Treatment of extracted seeds with concentrated sulphuric acid for 20 minutes was the second method used to render the seed coats permeable.

After-ripening of the embryo was accomplished by treating seeds, the coats of which had been made permeable at 1° or 5° C. in moist granulated peat moss, for three to five months.

Each of the two steps described above are essential for germination of *Tilia americana*. Treatment of the seed coat was ineffective unless followed

by after-ripening at low temperature. Similarly, low temperature treatment produced very few seedlings except when pre-treatment had rendered the seed coats permeable.

These methods were applied practically in two ways. The fruits were planted in flats in June or July. These flats were placed in a cold frame and mulched during the winter. Good seedling production occurred the following spring. If the temperature is too high (above 20° C.) when the fruits are planted, it might be well to shade the flats for the high temperature period. Such a summer planting delays the seedling production until after the second winter. The second practical method for seedling production consisted in planting extracted, sulphuric acid-treated seeds in a flat and placing in a mulched cold frame in January. Seedlings appeared the following April or May. The methods here reported for the extraction of seeds from fruits, however, would be difficult to apply on a commercial scale. Further work is needed to discover a practical means of extraction.

The capacity of the extracted seed to absorb water was greatly increased by sulphuric acid treatment. Although the intact fruit absorbed a great deal of water, very little water entered the seed coat, most of it remaining in the fruit coat or inside the fruit coat surrounding the seed.

Excised embryos from dry fruits or from fruits and extracted seeds which had been in moist granulated peat moss at low temperatures for two or three months acquired green color in their cotyledons and started to grow within three days. However, very little total growth took place. Embryos from sulphuric acid-treated seeds, which had received low temperature treatment, grew much more rapidly.

Catalase activity was found to increase as after-ripening progressed but there was no absolute correlation between the degree of activity and the completion of after-ripening.

Oven and sample planting tests as well as flat plantings of *T. tomentosa*, *T. platyphyllos*, and *T. cordata* indicated the same general trend as *T. americana*. However, *T. tomentosa* germinated very poorly throughout the tests while *T. platyphyllos* and *T. cordata* germinated more easily and with higher percentages than *T. americana*.

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PHYSIOLOGICAL AND CHEMICAL CHANGES PRECED- ING AND DURING THE AFTER-RIPENING OF SYMPHORICARPOS RACEMOSUS SEEDS

FLORENCE FLEMION

INTRODUCTION

Seeds of *Symphoricarpos racemosus* Michx., commonly known as snow-berry, are very resistant to germinative conditions. Pammel and King (9) report complete failure to obtain seedlings in a planting of these seeds while Adams (1) obtained 50 per cent germination after two years in the case of seeds planted out-of-doors in September. Experiments were undertaken to determine the reason for this delayed germination and to find methods of obtaining speedily, if possible, a larger percentage of seedlings. Preliminary trials in which the seeds were mixed with moist peat moss and placed at various low temperatures for varying periods of time showed that this method which is so efficacious in many rosaceous and other temperate zone species (2) is not effective for the production of seedlings of this species. Methods of obtaining a high percentage of seedlings much more promptly were developed and considerable information as to the mechanism of the dormancy of these seeds was obtained.

Dormancy in seeds may be caused by an inhibiting effect of the seed coat, by a rudimentary or partially developed embryo, or by a dormant embryo which requires after-ripening. It was found that the delayed germination in seeds of *Symphoricarpos racemosus* is due to a combination of these factors. It is not common to have all three of these factors involved in the dormancy of a seed. For germination to take place it is necessary to overcome the effect of the seed coat and to bring about after-ripening and complete development of the embryo. The former may be accomplished by soaking the seeds for 75 minutes in concentrated sulphuric acid or by placing the seeds in a moist medium for four months at 25° C. during which period the seed coat undergoes destruction by fungi (10). The dormancy of the embryo is broken by an after-ripening period of six months in a moist medium at 5° C. During this period at 5° C. the embryo enlarges and increases in moisture content and in catalase and peroxidase activity.

MATERIALS AND METHODS

The berries were collected when mature in October or early November in the vicinity of the Institute at Yonkers, New York. Immediately after collection, the seeds were freed of the pulp by the use of a Hobart mixer, washed well, and spread out in thin layers for drying. The seeds were then stored (at room temperature) in containers covered with cheesecloth.

When the seeds were to be placed in moist condition they were mixed with granulated peat moss which gives a good air supply and at the same time holds a large reserve of water. The peat moss was obtained from Atkins and Durbrow, New York City. Lots of 200 or 300 seeds in duplicate were used for all the experiments described except where otherwise indicated.

BREAKING DORMANCY

EFFECT OF LOW TEMPERATURE

As stated above, these seeds could not be induced to germinate by mixing in moist peat moss and placing at low temperatures for various periods. Results of these tests are summarized in Table I which show that no appreciable germination resulted in any of these experiments even though a number of conditions were tested and the treatments were continued for periods of several years. Experiments were then conducted in which the extremely tough seed coats were modified. It was found that very good germination would result if the seeds were then placed at low temperature for after-ripening and complete development of the embryo to take place. No germination occurred at temperatures above 10° C.

TABLE I

PERCENTAGE GERMINATION OF SYMPHORICARPOS RACEMOSUS SEEDS WHEN MIXED IN MOIST PEAT MOSS AND PLACED AT VARIOUS TEMPERATURES

Experiment started date*	Temperature	Percentage germination				
		1.5 years	2 years	2.5 years	3 years	4 years
December 5, 1928	1° C.	0	0.5	1.5	3	4
	1° and 10° C. alternated weekly	0	0**	—	—	—
	1° and 15° C. alternated weekly	0	0**	—	—	—
	5° and 10° C. alternated weekly	0	0**	—	—	—
	5° and 15° C. alternated weekly	0	0**	—	—	—
	10° and 25° C. alternated weekly	0	0**	—	—	—
June 7, 1929	1° C.	0	0	5	5	5
	5° C.	2	5	10†	—	—
	10° C.	0	0	0†	—	—
	20° C.	0	0	0†	—	—

* Lots of 200 seeds of the 1928 crop.

** Seeds moldy, experiment discarded.

† Experiment discontinued.

DESTRUCTION OF SEED COAT FOLLOWED BY LOW TEMPERATURE

With H_2SO_4 . Seeds were soaked in concentrated H_2SO_4 (dilute solutions penetrate and destroy the embryo) for periods ranging from five minutes to several hours, washed well, and subsequently soaked in a large quantity of water for an hour, mixed in moist peat moss, and placed at low temperatures. The data in Table II show the percentage germination obtained

from treatments of 30 to 75 minutes' duration in concentrated H_2SO_4 followed by a low temperature. The best germination was obtained from the 75-minute treatment followed by 5°C . A higher percentage germination does not result because the coats are not uniformly destroyed by this treatment. An examination of the seeds which failed to germinate showed incomplete destruction of the coats. Longer soaking in H_2SO_4 fails to give a higher percentage germination because some of the seeds are killed by such lengthened treatment. It is seen from Table II that 5°C . is better than 1° or 10°C . for the after-ripening of the embryo.

TABLE II

EFFECT OF SOAKING SEEDS IN CONCENTRATED H_2SO_4 UPON SUBSEQUENT GERMINATION IN MOIST PEAT MOSS AT LOW TEMPERATURES

Time in H_2SO_4 , minutes*	Percentage germination at low temperatures					
	6 months			9 months		
	1°C .	5°C .	10°C .	1°C .	5°C .	10°C .
75	12.3	44.5	4.0	17.3	55.8	6.6
60	5.3	32.8	6.3	11.0	48.0	11.6
45	7.6	15.7	0.6	13.6	44.8	5.6
30	0.6	4.0	1.0	1.0	7.8	5.6
0	0.0	0.2	0.0	0.0	0.3	0.0

* Lots of 300 seeds of the 1932 crop treated on November 4, 1932.

At 25°C . The effect of various periods at temperatures ranging from 15°C . to 35°C . upon the subsequent germination at 5°C . was studied. No germination occurs while the seeds are at these high temperatures. The results in Table III show that the efficacy of the treatment at 25°C . increases with the duration of the treatment up to four months. Periods longer than four months give poorer results. Treatments at 35°C ., 30°C ., and 20°C . are not as good as at 25°C . and periods at 15°C . and 10°C . are very poor in bringing about changes in the seed coats prior to the after-ripening and germination at 5°C . Pfeiffer (10) found fungi in the seed coat under the conditions prevailing at these temperatures and has studied the activity of fungi in the decomposition of the seed coat at 25°C . It is seen from Table III that the percentage germination resulting when the seed coats are modified by fungi and the embryos are after-ripened at 5°C . ranges from 25 to 80. This variation in the percentage germination obtained is probably due to the activity of destructive organisms in partially or completely destroying some of the seeds while at 25°C .

Experiments were conducted in order to determine whether seeds kept under moist conditions for three and four months at 25°C . in the absence of fungi would germinate after subsequent after-ripening at 5°C . Four lots of seeds were subjected to the following treatments: (1) five and one-half

hours in 5 per cent AgNO_3 followed by 5 hours in 5 per cent NaCl and then one hour in very dilute NaCl , (2) one hour in 5 per cent AgNO_3 followed by one hour in 5 per cent NaCl and washed with distilled water, (3) one-half hour in 0.5 per cent Uspulun followed by one-half hour in distilled water, and (4) thorough washing with distilled water. The seeds were then placed in sand in sterile tubes at 25°C . and watered with sterilized water as often as necessary. At the end of three and four months at 25°C . the seeds did not have the dark color and soft coats of those seeds which had been in moist peat moss (not sterilized) for the same length of time at 25°C . nor did they germinate when subsequently subjected to 5°C . Upon examination no injury in the use of distilled water or Uspulun could be detected (10) yet in no case did these sterilized seeds germinate after

TABLE III

EFFECT OF PREVIOUS PERIODS AT 25°C . UPON THE SUBSEQUENT GERMINATION IN MOIST PEAT MOSS AT 5°C .

Crop	Months at 25°C .	Date transferred to 5°C .*	Percentage germination at 5°C .**		
			4 months	5 months	6 months
1930	4	November 24, 1931	8.2	64.2	73.0
1932	1	December 5, 1932	0	0	0
	2	January 5, 1933	0.8	10.5	19.5
	3	February 4, 1933	14.8	49.5	63.5
	4	March 7, 1933	47.3	69.8	80.0
	2	April 16, 1933	3.8	7.8	13.0
	4	April 16, 1933	10.0	18.0	24.5
	3	February 24, 1933	15.7	41.1	50.4
	4	March 27, 1933	14.9	37.0	42.8

* Duplicate lots of 200 seeds each.

** These figures represent the percentage germination of seeds remaining intact throughout the experiment. A small number of seeds (2 to 15 per cent depending upon the length of time at the high temperatures) had to be discarded because they did not remain viable.

nine months at 5°C ., while those which had been in moist peat moss for four months at 25°C . gave about 65 per cent germination after six months at 5°C .

Combination of H_2SO_4 and high temperature. When seeds are treated with concentrated sulphuric acid for 75 minutes and placed at 5°C . about 50 per cent germinate after six months. When the seed coats are broken down by placing in moist peat moss at 25°C ., 25 to 80 per cent germination can be obtained. It was found that if a combination of the sulphuric acid and high temperature (25°C . or 30°C .) treatments were used, germination of 60 to 90 per cent resulted.

Seeds were soaked in concentrated H_2SO_4 for various periods, washed

well, soaked in water for one hour, mixed in moist peat moss, placed at high temperatures for two weeks or one month, and then transferred to 5° C. The percentage germination not shown in tables obtained by this method ranges from 60 to 90. Seeds which had been soaked in H_2SO_4 for 75 minutes and were subsequently placed at 25° C. for two weeks gave 66 per cent germination (Table IV) while the seeds which were treated with H_2SO_4 for only 45 minutes gave better germination if followed by one month at 25° C. rather than two weeks. Pfeiffer (10) found that during this short period of two weeks or one month at 25° C., the remaining part

TABLE IV

EFFECT OF TREATING SEEDS WITH CONCENTRATED H_2SO_4 AND HIGHER TEMPERATURES BEFORE PLACING AT LOW TEMPERATURES UPON SUBSEQUENT GERMINATION AT 5° C.

Treatment		Percentage germination at 5° C., months			
Time in H_2SO_4 , minutes*	Time at 25° C., months	3.5	4	5	6
75	1	14.5	36.2	44.0	46.2
75	0.5	3.6	25.3	60.7	65.8
75	0	4.5	11.0	36.2	46.3
60	1	10.3	27.6	62.8	68.2
	0.5	7.3	31.2	52.5	58.3
	0	1.2	6.7	34.3	47.0
45	1	4.7	23.8	51.8	59.2
	0.5	1.0	7.6	38.0	50.2
	0	0.3	1.8	6.6	14.7
0	4	4.6	10.0	24.5	31.8

* Duplicate lots of 300 seeds treated and placed at 5° C. on April 16, 1933.

of the coats which had not been destroyed by H_2SO_4 were thoroughly infected with fungi. Apparently the fungi were efficacious in the case of seed coats not completely destroyed by the acid and were not injurious (at least within two weeks) to those seeds, the coats of which had been sufficiently destroyed.

CHANGES ASSOCIATED WITH THE BREAKING OF DORMANCY

Studies were made on the changes taking place in seeds undergoing various treatments which bring about subsequent germination. Embryos were excised from seeds which had been subjected to various conditions conducive to the breaking of dormancy. The excised embryos were placed in aerating water (water through which air was continuously passed) for one month. The embryos (magnified 4X) before and after aeration are shown in Figure 1. The embryos from the dry seeds and from seeds which had been in moist peat moss for two and one-third months at 5° C. or for

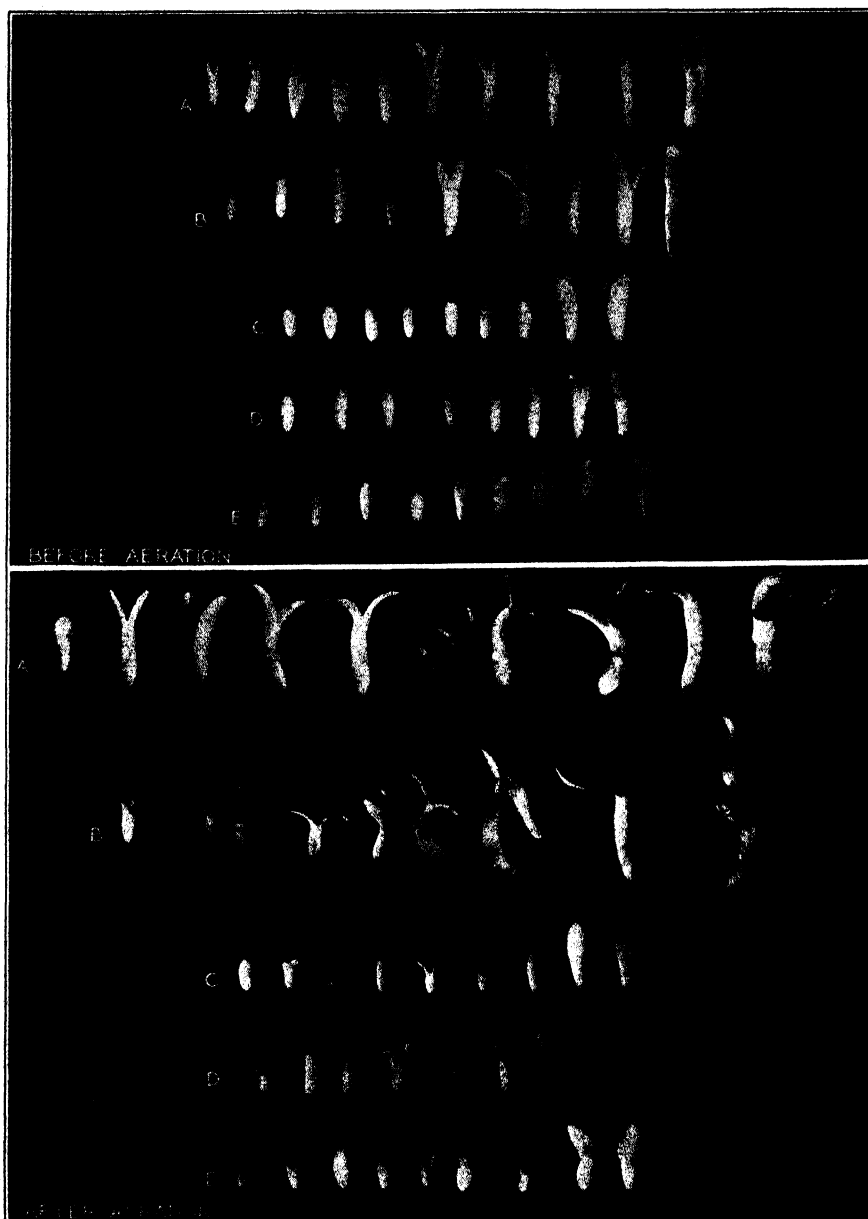


FIGURE 1. Excised embryos (magnified $4\times$) of *Symphoricarpos racemosus* seeds which had been subjected to various treatments. (A) Soaked 75 minutes in concentrated H_2SO_4 followed by two and one-third months at 5°C . in moist peat moss; (B) at 25°C . in moist peat moss for three months, then transferred to 5°C . for two and one-third months; (C) at 25°C . for four months in moist peat moss; (D) at 5°C . for two and one-third months in moist peat moss; (E) embryos from dry seeds.

four months at 25° C. remained practically unchanged during the aeration period. However, the embryos of seeds which had been in moist peat moss for three months at 25° C. or treated for 75 minutes in concentrated H₂SO₄, and subsequently placed at 5° C. for two and one-third months, had sufficiently after-ripened to show considerable development when placed under conditions furnishing an adequate oxygen and water supply. The period of three months at 25° C. was effective in overcoming the effect of the seed coat but did not materially alter the embryos. The period of two and one-third months at 5° C. partially after-ripened the embryos especially when the coats had been previously destroyed with either sulphuric acid or high temperature.

Enzyme Activities

The enzyme changes occurring during the breaking of dormancy were also studied. For catalase determinations a definite number (100 or 200) of seeds were ground in a mortar with pure quartz sand and 0.5 gram of calcium carbonate, washed into a 50 cc. volumetric flask, and made up to volume. Aliquots of 5 cc. were drawn for the determinations. The procedure and apparatus as described by Davis (3) were used except that the hydrogen peroxide was neutralized with calcium carbonate instead of sodium hydroxide. Peroxidase activity was determined by using aliquots of the same solutions used for catalase. The method used consisted of the oxidation of pyrogallol to purpurogallin under controlled conditions and the subsequent colorimetric determinations of the amount of purpurogallin formed (7).

The data show (columns 4 and 5 of Tables V and VI) that while kept at 5° C. the seeds increase in catalase and peroxidase activity several

TABLE V

EFFECT OF VARIOUS TEMPERATURE TREATMENTS UPON DRY WEIGHT, MOISTURE CONTENT AND ENZYME ACTIVITIES

Treatment	Dry Weight per 300 seeds grams	Percentage moisture	Catalase cc. of O ₂	Peroxidase mg. of purpurogallin	Percentage germination after 6 months at 5° C.*
Dry seeds	2.764	4.66	7.2	4.84	0
4 months at 5° C.	2.732	33.93	16.8	14.96	0
1 month at 25° C.	2.670	34.89	9.4	6.30	1.0
2 months at 25° C.	2.364	40.21	6.0	—	19.6
3 months at 25° C.	2.435	39.08	6.4	—	12.3
4 months at 25° C.	2.168	44.45	4.5	4.29	31.8
3 months at 25° C. followed by 2½ months at 5° C.	2.080	49.46	29.7	13.87	63.7

* Duplicate lots of 300 seeds.

hundred per cent. These results agree with other determinations of catalase and peroxidase activity during after-ripening (4, 5, 6, 8). No increase in the activities of these enzymes occurs during the preliminary period at 25° C. while the seed coats are undergoing decomposition, in fact there is a slight decrease. It is of interest, too, that when seeds are kept at 5° C. without any previous treatment to destroy the seed coats, an appreciable increase in enzyme activity results although such seeds will never germinate, nor do excised embryos of such seeds develop seedlings when placed in aerating water.

TABLE VI

DRY WEIGHT, MOISTURE CONTENT, AND ENZYME ACTIVITIES OF SEEDS TREATED WITH CONCENTRATED H_2SO_4 AND KEPT IN MOIST PEAT MOSS FOR TWO AND ONE-THIRD MONTHS AT 5° C.

Duration of H_2SO_4 treatments, minutes*	Dry weight per 300 seeds grams	Percentage moisture	Catalase cc. of O_2	Peroxidase mg. of purpurogallin	Percentage germination after 6 months at 5° C.
75	1.610	42.06	34.8	18.6	45.3
60	1.872	35.12	21.4	17.1	40.2
45	1.915	34.05	19.7	11.9	15.3
30	2.069	32.03	16.7	13.1	6.3
0	2.636	32.00	10.0	—	0
Dry seeds	2.764	4.66	5.5	4.8	—

* Treated and placed at 5° C. on February 6, 1933.

Water Relations

Uptake of water. Determinations of the moisture content of the seeds under various conditions (some of the data are given in Tables V and VI) have shown that the dry seeds take up water up to about 30 per cent very readily. Additional water is taken up slowly at both high and low temperatures. Although the data in Table VI seem to show some relation between the moisture content and germination, the data as a whole do not indicate a close relationship since seeds kept under conditions not favorable for after-ripening often take up considerable additional moisture. Further experimentation is necessary before any definite conclusions can be drawn as to the relation between the uptake of water and the subsequent germination of the seeds.

VITALITY DURING STORAGE

Room temperature storage. Changes take place as the seeds age under ordinary storage conditions at room temperature which are reflected in the germination resulting after various treatments. Experiments were conducted in which the seeds were treated for 45 minutes with concentrated H_2SO_4 and placed for nine months at 1° C. The change in response obtained with seeds after varying periods of storage for the crops of three

years is shown in Figure 2. It is seen that the freshly-harvested seeds do not respond nearly so well as do the seeds after a period of three to seven months of storage at laboratory temperature. After the peak at three to seven months the per cent germination obtained from subsequent lots gradually became less until, after 12 months, very low germination resulted.

These experiments do not show, however, that the seeds lose their vitality under these storage conditions. In fact, two and one-half year old

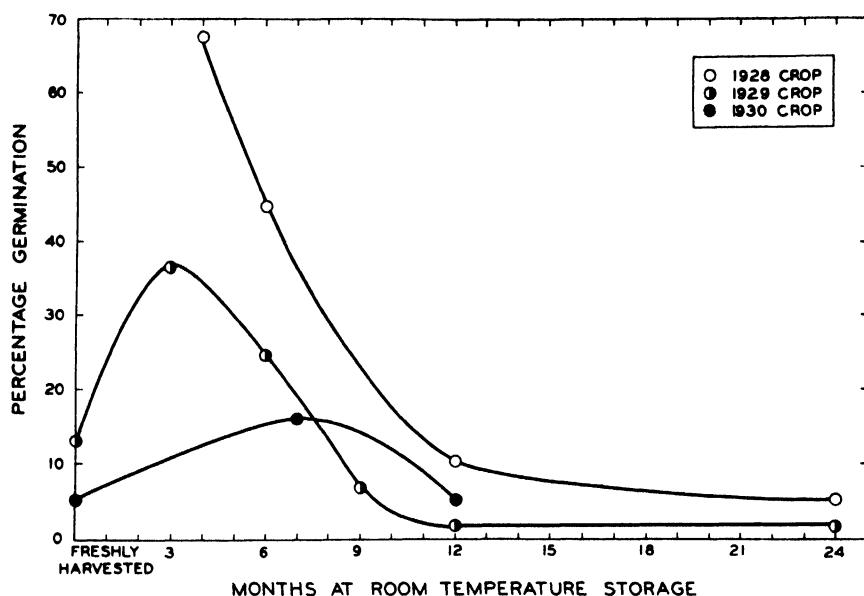


FIGURE 2. Curves showing changes in the response of *Symphoricarpos racemosus* seeds to a suboptimal treatment (45 minutes concentrated H_2SO_4 followed by nine months at $1^\circ C.$) at various periods after harvest. These results are not due to a loss in vitality but are probably caused by alterations in the seed coat during dry storage.

seeds of the 1930 crop which failed to give any appreciable germination after 45 minutes of concentrated H_2SO_4 and nine months at $1^\circ C.$ responded well to a more favorable treatment (60 minutes concentrated H_2SO_4 and two weeks at $30^\circ C.$ followed by six months at $5^\circ C.$). These experiments show, therefore, that storage at room temperature markedly affects the character of the seed coat so that a treatment employed for the destruction of the coat may not be optimal for seeds of all ages.

The length of time in which the embryos remain viable under these storage conditions has not yet been determined. Seeds two and one-half years old are still viable.

Effect of storage at various relative humidities. In order to determine the effect of relative humidity on the vitality of seeds, seeds were kept at room temperature in desiccators over various concentrations of H_2SO_4 (11) such as were required to give the humidity desired. The controls were also kept in desiccators. After two years of storage under these conditions the seeds were removed from the desiccators, treated for 45 minutes in concentrated H_2SO_4 , and placed at an ice chest temperature (a fluctuating temperature of 2° to $8^\circ\text{C}.$) for after-ripening. The germination resulting after four, six, and eight months at this temperature is shown in Table VII. It is seen that seeds stored at about 25 per cent relative humidity gave about the same percentage germination as did the controls. Seeds stored at the other relative humidities germinated very poorly. Conditions prevailing at the humidities above and below 25 per cent relative humidity were injurious to the seeds.

TABLE VII

EFFECT OF ROOM TEMPERATURE STORAGE OF SEEDS AT VARIOUS HUMIDITIES FOR TWO YEARS UPON SUBSEQUENT GERMINATION AT ICE CHEST TEMPERATURES (2° TO $8^\circ\text{C}.$ FLUCTUATING)

Storage condition	Percentage germination, months*		
	4	6	8
CaO	0	0	0.5
Conc. H_2SO_4	0	5	5.5
55.58% H_2SO_4 (about 25% humidity)	14.5	47	61.5
43.13% H_2SO_4 (about 50% humidity)	4	11	12
30.2% H_2SO_4 (about 75% humidity)	0	0	0
Control	8	38	57

* Each lot of 200 seeds was treated for 45 minutes with concentrated H_2SO_4 before being placed at the low temperature.

SEEDLING PRODUCTION

It is possible to obtain a good percentage of seedlings if seeds which have been almost completely after-ripened by one of the methods described in a previous section of this paper are transferred from the low temperature and planted in greenhouse flats.

The production of seedlings of *Symphoricarpos racemosus* seeds was also attempted under conditions more nearly approaching regular greenhouse practice. The seeds were planted in flats which were kept at various controlled temperatures or placed out-of-doors in cold frames, either open, covered with a board cover, or mulched and covered with a board cover. In these experiments the highest percentage of seedlings occurred when the seeds were planted in flats and placed outside in the spring in a cold frame which received a board cover the following winter. From 500 seeds

planted thus, 71 per cent of seedlings resulted the following spring. When planted in flats out-of-doors in the fall only 15 to 18 per cent germinated the following spring and only an additional few germinated the second spring. In the experiments in which the flats were placed at various controlled temperatures the highest percentage obtained (46 per cent) resulted after three months at 21°C. followed by five months at 5°C.

In these experiments, too, the seeds responded to a period at high temperature followed by low temperature. An examination of seeds kept in soil in flats out-of-doors during the summer months showed the presence of fungi (10). The high temperature during the summer is conducive to the decomposition of the seed coat and the subsequent cold winter months after-ripen the embryos sufficiently so that germination occurs the following spring.

SUMMARY

In order to induce germination in seeds of *Symphoricarpos racemosus* it is necessary that the seed coat be disintegrated. This can be accomplished by placing the seeds for a period of three or four months in moist acid peat moss at 25°C., or by soaking the seeds in concentrated H₂SO₄ for 75 minutes, or by H₂SO₄ treatment and several weeks at 25°C. After the required changes in the seed coat have occurred it is still necessary to after-ripen the embryo which can be brought about by a period of six months at 5°C. Of these three methods which modify the seed coats, the combination of H₂SO₄ treatment and a short period at 25°C. is the best and germination percentages of 60 to 90 may be obtained in this way.

For the production of seedlings on a large scale the best method is to plant the seeds in flats in spring and place out-of-doors in a cold frame which is covered with a board cover during the winter months. Germination occurs the following spring. In nature, as in our laboratory experiments, the seeds respond to a high temperature followed by low temperature. During the period at high temperature (summer months) conditions are favorable for the modification of the seed coats; the embryos are then after-ripened during the subsequent cold winter months.

That the seed coats undergo changes during dry storage at room temperature is shown by the fact that a suboptimal treatment of sulphuric acid followed by low temperature produced a maximum germination from seeds which had been stored about three months. Practically no seeds stored nine months or longer germinated although they were shown to be still viable when subjected to a more effective treatment.

The seeds increased several-fold in catalase and peroxidase activity during the period of after-ripening at 5°C. When at 25°C. the activity of these enzymes does not increase but there is instead a slight decrease.

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MORPHOLOGY OF THE SEED OF SYMPHORICARPOS RACEMOSUS AND THE RELATION OF FUNGAL INVASION OF THE COAT TO GERMINATION CAPACITY

NORMA E. PFEIFFER

Earlier accounts dealing with the seed of the snowberry (*Symphoricarpos racemosus* Michx.) have been concerned with the structure of the coats without relation to their chemistry or their functioning. Results with treatment of seed in order to obtain speedier germination (3) suggested the need of more careful microscopic and chemical examination with relation to this particular problem.

HISTORICAL

Two views as to the origin of the outer hard layers of the mature seed have been emphasized in previous descriptions. These sclerified cells are supposed to originate, like the layer of barely distinguishable parenchyma cells within, from the single integument (4, 5). Or the stone is considered to be that of a drupe, derived from three distinct layers, the inner epidermis, the exodermis, and an external zone, that is, from parts of the ovary wall (8). This view is somewhat modified in a general account of the family, Caprifoliaceae, where the integument is described as being compressed, except for its inner epidermis, which gives rise to a "mantle layer" (6). Although the nucellus disappears early and completely, a cuticle between this tissue and the integument is preserved. The seed coat in *Symphoricarpos* is specifically described as having a strongly pitted and thickened outer epidermis, under which there are two layers of highly refractive fibers, lying at right angles to each other.

The present work tends to confirm the view that these two fibrous zones, along with a single less thickened outer layer of cells, arise from the ovary wall. The integument is represented at maturity by the very striking outer epidermis, rather than the inner, and by a number of layers of crushed parenchyma cells, while the nucellus has disappeared. There are two cuticles; the finer one occurs between the outer epidermis of the integument and the innermost fibers, and the second, which is much more readily observed, is between the mature endosperm and the parenchyma of the old integument. The development of the tissues and their chemistry are considered along with their functioning.

METHODS

Seeds of *Symphoricarpos racemosus* were studied both by means of fresh and prepared material. Free-hand sections and those obtained by

the use of the freezing microtome were used in the case of the fresh ovaries and seeds for the study of both structure and chemistry of the parts. Preparations of similar stages imbedded in paraffin, either by the usual method in which cedar oil served as a clearing agent, or by the use of butyl alcohol (11), were used for more detailed study of the morphology.

In the microchemical work, the standard tests (1) for the different substances were used.

MORPHOLOGY OF MATURE SEED COAT

Although in the strict sense of the word, the seed includes only the structures derived from ovule tissues, convenience sometimes includes under the term certain layers which are derived from the ovary wall. This has not been emphasized as the situation in *Symphoricarpos* by previous

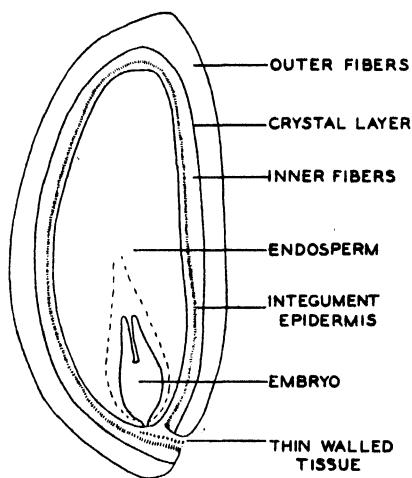


FIGURE 1. Diagram of median longitudinal section of *Symphoricarpos racemosus* seed. Magnification $\times 16.5$.

investigators. Therefore it should here be made clear that the word seed is used to include the hard enveloping layers which at maturity are not readily separated from the tissue derived from the integument, but are easily separated from the fleshy portion of the pericarp.

Ripe seeds of the variety used average from 4 to 4.6 mm. in length, and are about 2.5 mm. wide and 1.5 mm. thick. They are decidedly convex on one side, almost plane on the other, and are creamy in color. The outer hard coat is relatively thick ($250-275\mu$) (Fig. 1). The cells of the outermost layer are large with somewhat thickened walls, which are pitted. Within this layer are two readily distinguished zones of pitted fibers, long, pointed, and with heavily thickened walls (Fig. 2). In the wide outer zone, the long

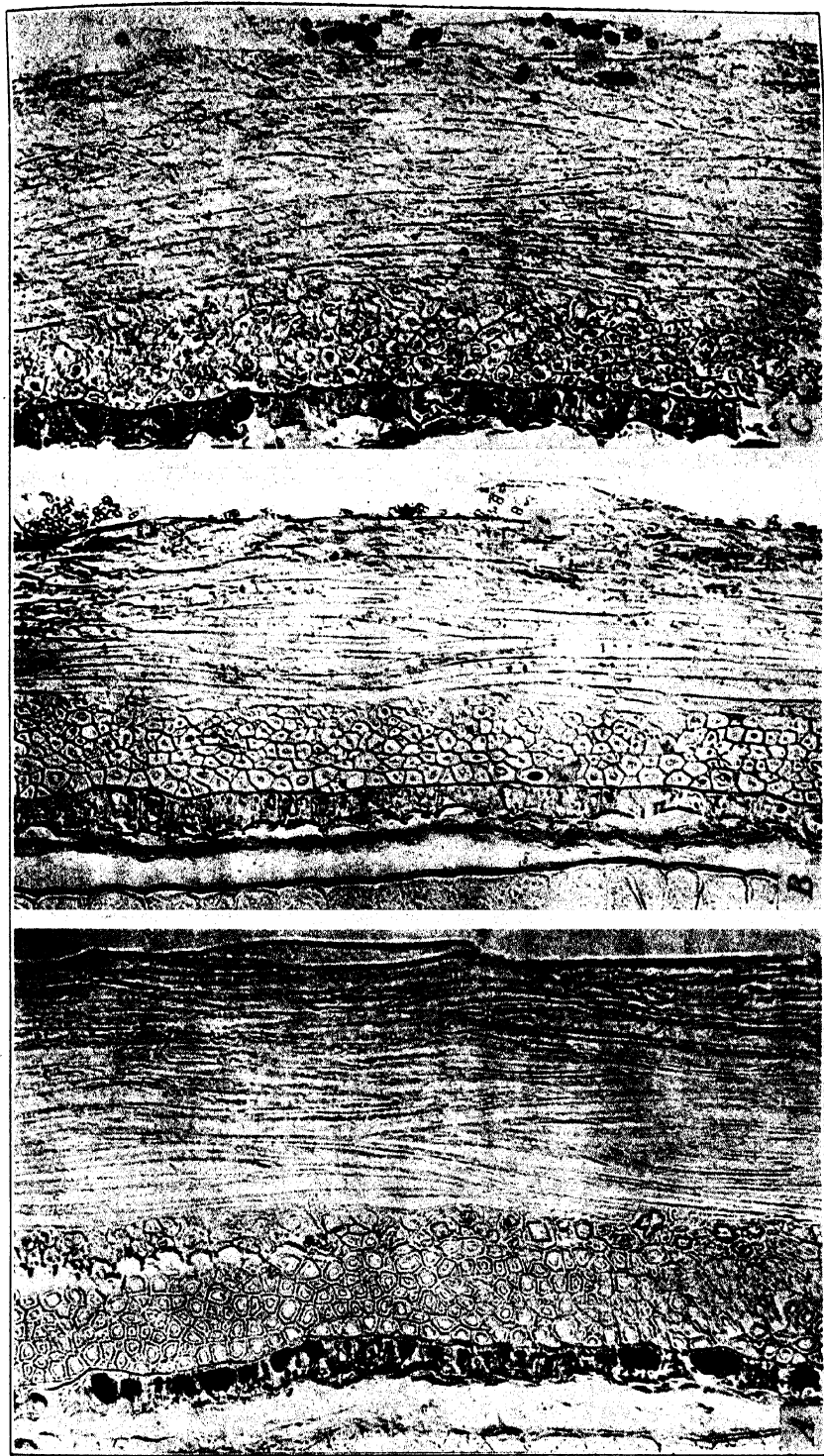


FIGURE 2. *Symphoricarpos racemosus* seed coat. A. Dry seed; integument epidermis, inner fibers, crystal layer, outer fibers. B. Seed in moist peat moss at 25° C. for three months; note thinner coat, thinner walls of fibers, fungus structures; *l* = two-celled fungus bodies; *c* = fungus thread in fiber. C. Seed in moist peat at 35° C. for three months; *b* = brown fungus bodies. Magnification X 200.

axes of the fibers lie more or less parallel to the long axis of the seed, while in the narrow inner zone, the long axis is at right angles to this direction. Hence one sees the long view of one group of fibers and the cross view of the other in any median section of the seed. There is a single layer of cells between these two fiber zones in which the individual cell is isodiametric and is conspicuous because of the occurrence of single large monoclinic crystals of calcium oxalate.

Closely appressed to the inner fibers is a single layer of cells which stands out sharply because of brown color of contents and thick wall (Fig. 2). In surface view, these cells have irregular wavy outlines much like those of mature epidermal cells of leaves. This readily stained layer lies alongside the inner fibers to the restricted placenta region, where the fibers are discontinuous. On its inner side lie several crushed layers of cells (Fig. 2 A and B), through which the path of a fibro-vascular bundle can be traced from the placenta region almost if not quite encircling the endosperm. There is a distinct membrane between this crushed tissue and the endosperm (at the left in Fig. 2 B). The relatively small stout embryo is imbedded in a cleft in the endosperm (Figs. 1 and 5 O).

CHEMISTRY OF MATURE SEED

The thick walls of the fibers are relatively hard at maturity. Microchemical tests made to determine the components showed the presence of lignin, pentosans, and cellulose. The first was readily demonstrated by means of phloroglucin-HCl, which gave the characteristic red-violet color for lignin, and by solubility tests; in sections treated with 50 per cent chromic acid, the walls dissolved in a short time, while in copper-oxide-ammonia solution, thick walls were evident after three days. After washing these with 5 per cent acetic acid, the wall substance still gave a positive test for lignin.

Sections were treated with potassium chlorate, hydrogen peroxide, and nitric acid as oxidizing agents to remove the lignin from fiber walls. Of these, the last was the most successful. After complete oxidation of the lignin, as shown by a negative test with phloroglucin-HCl, these same preparations were gently heated, with the result that the characteristic cherry red color of pentoses appeared. This was further tested by treating similar sections with orcin, followed by a drop of concentrated hydrochloric acid. Absence of lignin was indicated by failure to color violet, while gentle heating produced a blue color, with some blue-green precipitate, which corroborated the finding of pentoses. These products of hydrolysis show the presence of the pentosans, araban and xylan.

Sections from which the lignin had been removed by means of nitric acid when carefully washed and tested with iodine-potassium-iodide and

75 per cent sulphuric acid, gave the blue color characteristic of cellulose membranes.

The brown layer next within the fibers contains the same components except that it is cutinized as well as more highly lignified. The color reaction with phloroglucin-HCl is much deeper than in the fibers, and longer time is required to remove the lignin here either by solution in 50 per cent chromic acid or by oxidation in nitric acid. After such removal, the cuticle on the outer side stained red with Sudan III.

The crushed layers of cells within this layer have cellulose walls. They are separated from the endosperm by a very distinct cutinized membrane which in surface view shows the polygonal outlines of cells.

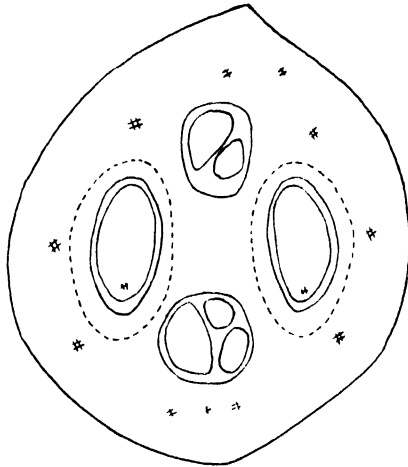


FIGURE 3. *Symphoricarpos racemosus*. Diagram of cross section of ovary, with two sterile chambers containing several ovules, two fertile chambers each with one ovule; inner layers of ovary wall already differentiating about the fertile chambers. Magnification $\times 56$.

The endosperm is composed of relatively large cells with thin walls made up of cellulose and a thin hemicellulose layer on either side. The outermost layer of cells appears somewhat differentiated both in size and contents.

The embryo is relatively small about one mm. long as an average and thin walled. The organs are differentiated, but not much developed.

DEVELOPMENT OF SEED

Seed coat. The structure of the ovary in *Symphoricarpos* is distinctive in its symmetrical arrangement of four chambers, two of which regularly produce several ovules, and the other two but a single ovule (Figs. 3 and 4 D). In the course of development, the former abort, and the larger single ovules, with luck in pollination, produce the fertile seeds. As a result, a

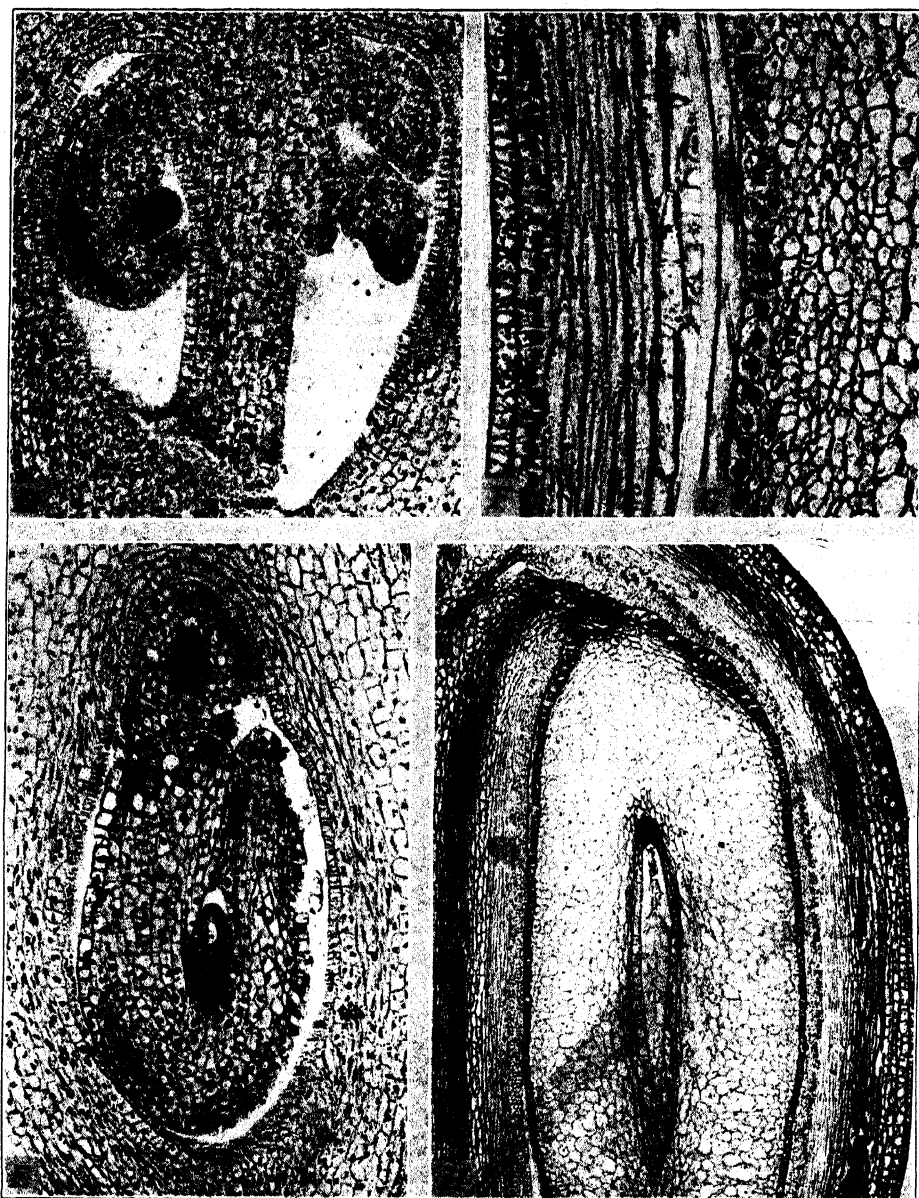


FIGURE 4. Development of *Symphoricarpos* seed. D. Longitudinal section of young ovary; in right chamber, sterile ovules, in left fertile ovule with nucellus and single integument. $\times 136$. E. Anatropous ovule; integument and nucellus layer surrounding megaspore mother cell. $\times 136$. F. Longitudinal section of young ovary wall; left to right, innermost layer, crystal layer, outer fibers. $\times 330$. G. Cross section of similar portion of ovary wall. $\times 330$. H. Longitudinal section of part of young fruit; few-celled embryo; endosperm; massive integument with little differentiated inner epidermis and conspicuous outer epidermis terminating at short funiculus (composed of thin-walled cells) surrounded by developing stony and fleshy layers of pericarp. $\times 42$.

cross section of the ovary at maturity shows two conspicuous cavities with a single large seed in each, alternating with the two much appressed, almost slit-like cavities bearing the remains of little brown dried ovules. Since basal growth is greater, the lower part may show only the seed cavities.

In the very young stages, where the ovules on their short stalks have not yet assumed the anatropous position (Fig. 4 D) there appears to be little structural difference between the ovules or the ovary wall about the chambers. Although the ovules differ in size, at the megaspore mother cell stage there is in each a single delicate nucellus layer covering the mother cell and a single massive integument in the process of development (Fig. 4 E). The innermost layer of cells of the ovary wall is tabular in appearance in longitudinal view (Fig. 4 E and F); cells of the adjacent layer are larger in dimensions. Soon, in the case of the fertile chambers, very small monoclinic calcium oxalate crystals appear in this second layer (Fig. 4 F and G), first in the region toward the outer side of the ovary, later toward the center. These crystals increase in size with the development of the organ. Inasmuch as cellular division has not been completed at the time of their first appearance, it is not unusual to see the nuclear spindle with a fairly conspicuous crystal occupying most of the rest of the cell. No such crystals occur about the sterile chamber. Other calcium oxalate crystal masses of the druse type occur in the parenchyma of the ovary wall (Fig. 5 L), and later in integument cells.

A cuticle covers the surface layers in this young structure, finer about the lining of the ovary chamber, a little heavier about the nucellus and the integument surface. The walls of basal cells of the micropyle are also cutinized. The outer epidermis of the integument becomes differentiated in contents as well as wall. In fresh material at the flowering stage it is yellowish-brown in both fertile and sterile ovules. In prepared slides, it stains very densely up to the region of the funiculus, where the modifications in the wall are not so marked although the contents appear different. The inner epidermis of the integument is somewhat differentiated from the intervening 12 to 16 parenchyma layers, in its larger cells and larger nuclei and in the presence of the inner cuticle.

The nucellus cells which surround the embryo sac of the usual eight-nucleate type, with egg apparatus, polar and antipodal nuclei, are difficult to distinguish at this stage, so that one easily understands early failure to distinguish them.

After fertilization, changes occur relatively rapidly along with great growth of the ovule and ovary wall especially in the lower portion. The nucellus disappears and the parenchyma cells of the integument lose their contents and are gradually flattened by the pressure of other active parts (Figs. 4 H and 5 K, M, N). At an intermediate stage, where but half of

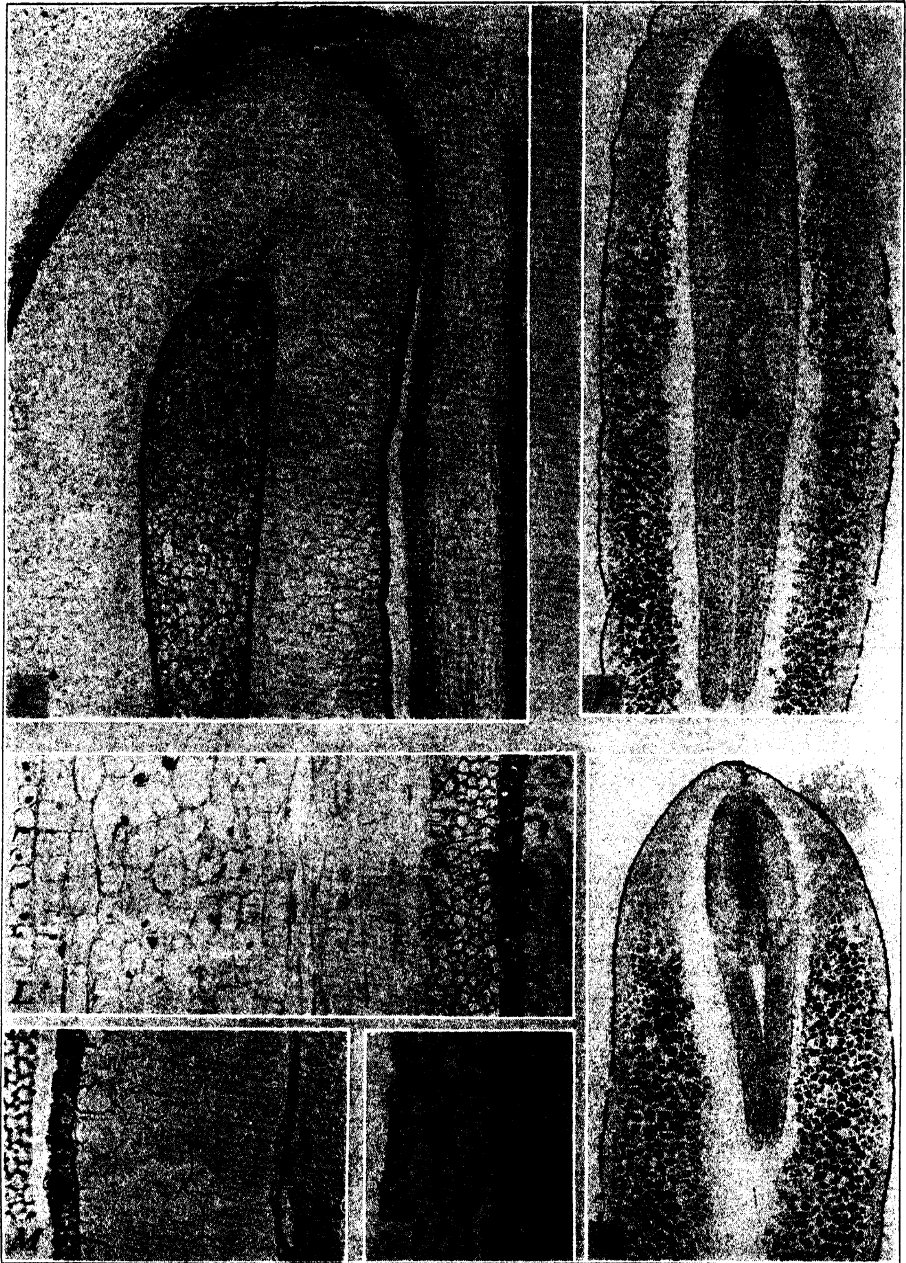


FIGURE 5. Development of *Symphoricarpos* seed. K. Intermediate stage; spherical embryo; endosperm; integument crushed inner and turgid outer parenchyma, epidermis; right, pericarp. $\times 45$. L. Pericarp of K; left to right, fleshy layers, epidermis of "stone," outer fibers, crystal layer, inner fibers, outer cuticle, integument epidermis and parenchyma. $\times 144$. M. Older stage; inner fibers, integument epidermis and crushed parenchyma; inner cuticle; endosperm. $\times 144$. N. Later stage; same tissues except fibers. $\times 144$. O. Embryo in mature seed; endosperm; inner cuticle. $\times 32$. P. Embryo after-ripened at low temperature. $\times 32$.

the original twelve to sixteen parenchyma layers remain turgid, the inner epidermis of the integument is still recognizable (Fig. 5 K). The outer epidermis is much changed by the deposition of pentosans and lignin, mostly on the inner and side walls, and by the accumulation of oil and tannin-like substances. These modifications occur earlier than those in the ovary wall, and growth of the ovule tissues brings them into such close contact with the wall that it is difficult to separate the integument epidermis from the inner pericarp cells.

In the longitudinal section of the young ovary, the innermost layer of the wall appears to be made up of tabular cells (Fig. 4 E and F) which are, however, elongated in the plane seen in a cross section of the ovary (Fig. 4 G). The nucleus is also elongated in this plane. At first the nuclear spindles are so oriented that the cell walls resulting from division are radial in position. Later, pressure results in oblique walls (Fig. 4 G) partly through shifting in growth of the cells already formed and partly through the orientation of later nuclear spindles. Transverse sections of the ovary now show these to lie with one pole slightly higher than the other, and with the equatorial plate approximately parallel with the long axis of the cell. In this fashion, division and development of the original simple layer result in three layers of much elongated cells with pointed ends and with their long axis lying in the plane of the cross section of the ovary. Farmer (2) has described a similar development of the innermost pericarp layers in *Sambucus nigra*, except that he reports the result due entirely to the overlapping of cells of which the pointed ends slip over each other, with no tangential divisions.

In the cells next to the innermost layer, crystals of calcium oxalate (Fig. 4 F and G) appear early, as already indicated. These increase greatly in size, and eventually with the disintegration of the living material, almost fill the cell. At the intermediate stage described above, the nucleus has disappeared in these cells (Fig. 5 L).

In the differentiation of the pericarp, the stony layers come from these two innermost layers as well as from fibrous cells derived from layers of elongated cells, about three in number at the megaspore mother cell stage of the ovule, but increased to eight or ten by the time of maturity of the embryo sac. Originally of cellulose, the walls of these cells show thickening with deposition first of pentosans, later of lignin. The thickening appears first in the innermost zone, derived from the innermost layer of the ovary wall, later in the outer zone. All become pitted. As seen in the mature fruit, a single layer of the pericarp outside these fibers is less modified as to form and wall thickening, but is also pitted (Figs. 2 A and 5 L). This layer usually adheres to the stony part when the pulp is removed.

Endosperm. The endosperm develops rapidly into the chief storage tissue, with slight differentiation of the superficial layer (Fig. 5 M, N, O).

All walls are relatively thin, except the outermost in this layer; they are composed of cellulose and a thin layer of hemicellulose. The contents are principally oil and globulin protein. The former is readily determined by means of its different refractive index and staining ability with Sudan III. The protein gives a vigorous biuret reaction, colors yellow with iodine-potassium-iodide, and vermilion red with Millon's reagent. It crystallizes readily in picric acid or in glycerine; the crystals so produced may be stained pink with eosin.

Embryo. Division of the fertilized egg gives rise to an embryo which is small in proportion to the amount of endosperm and the total size of the seed. An intermediate stage, after the thickening of pericarp cells has begun, shows a more or less spherical embryo with a conspicuous suspensor of five to seven cells (Fig. 5 K). Later the regions become differentiated and so oriented that in a median longitudinal section of the stone cut at right angles to the more nearly plane surface, the embryo appears with the radicle region directed toward the placenta, a short hypocotyl with the two cotyledons and an undeveloped stem tip at the opposite end (Fig. 5 O). The cotyledons are approximately as long as the rest of the embryo; the whole is compact and appears relatively stout in the longitudinal plane at right angles to this. Embryos are not of uniform size and show a wide range in any lot. Those in the basal fruits of an inflorescence tend to average larger than those in apical fruits, possibly due to longer time for development.

EFFECT OF EXPERIMENTAL TREATMENT ON THE SEED

The results of Flemion (3) in her attempts to get speedier germination of snowberry seeds by keeping in moist media under different temperature conditions and with treatment by means of sulphuric acid, directed the attention of the writer to the effect of such treatments on the seed coat. The temperature relations seemed especially interesting, inasmuch as she found a low germination percentage (5 to 10 per cent germination in two and a half years) at 1° to 5°C., but 75 to 80 per cent germination if the treatment in low temperature were preceded by holding in a moist medium at a higher temperature, 25° to 30°C., for four months. The use of sulphuric acid as a preparatory step or its use combined with a short interval in the higher temperature, followed by keeping at the low temperature, also produced the desired result, with germination percentage from 75 to 100 per cent in the most favorable combinations. The medium in these experiments was acid peat moss.

Under ordinary working conditions in germination experiments, due precautions are taken that there is no injury resulting from molds or other fungi, but no especial effort is made to rule out the presence of fungi in the medium. This obtains both in tests in peat moss and in soil.

Temperature. The first microscopic examination was made of the pericarp of snowberry seeds of the 1931 crop held in moist peat for one, two, three, or four months at 25°C., for three months at 30°C., and for four months at 5°C.; dry seeds were also used for comparison. Sections made with the freezing microtome were stained lightly with resorcin blue, anilin blue, ruthenium red, methylene blue, or Sudan III, and mounted in fructose jelly, in efforts to determine structural relations. It was soon found that fungi were present in the fibrous cells of the coats of all seeds kept at the higher temperatures, in less degree in those held for a short time as compared with those treated for a longer interval (Fig. 2 B and C). Hyphae and resting bodies of two sorts were found in the lumen of the fibers. The progress in the series seemed to be from the periphery inward, but not uniform in all regions of the seed.

Certain changes in the walls appear to be associated with the penetration of the fungi. Originally strongly doubly refractive in polarized light, they become relatively inactive as fungi develop. They become thinner, softer, so that they are easy to cut and the pits become much more conspicuous (Fig. 2 B and C). Originally very light in color, they are now brownish, so that regions where fungi have been active can be located as small dots or streaks on the whole seed, as long as there is little infection. When there has been much, the whole coat is brown and subject to collapse, probably because of loss of wall components. When the fungi penetrate the layer between the two fiber zones, the crystals of calcium oxalate disappear.

These findings were confirmed by study of many seeds of other series in peat, including similar tests of the 1932 crop with a wider range of temperature (20° and 35°, as well at 25° and 30°C.). It was found that the development of fungi was favored by the increased temperature, and that the penetration was greater with long intervals of time, up to a certain stage. Eventually, however, in three months or more, there was a sort of clearing effect in the fibers. This was due in part to the loss of material from the fiber walls, now much thinner, but also doubtless to a change in the fungi. The change included actual disappearance of hyphae and appearance of the compact bodies which are often seen, especially near the surface. Then, too, superficial fibers often disintegrated, leaving fewer heavily infected cells. With the highest temperatures used in the longer intervals, there were sometimes irregular cracks through the fiber zones and even through the original outer epidermis of the integument. Usually there was no development of fungi either within the cells of this epidermis or in the region of crushed integument parenchyma. When infection did occur in the latter region, entrance seemed to be through the placenta tissue rather than through the outer cuticle and cells of the

epidermis. In such cases, the effect on the percentage of germination is probably disadvantageous.

In contrast to the activity in the higher temperatures, material at 5°C. showed no invasion of fungi. Walls of fibers retained their original hardness, thickness, and color; they were doubly refractive in polarized light.

Examination of seeds placed in moist peat at 30°C. showed that fungi soon invaded the surface layer of cells. In the first nine days, no hyphae were seen within the cells, but in fifteen days, it was possible to see them as deep as the third layer of cells from the surface. Thereafter progress was relatively speedy.

Because of the general presence of fungi in the coat when moist peat moss was used as a medium for the seeds, series in soil were also examined. One set of seeds kept in soil in flats in the greenhouse for a year and others in flats outdoors for two, four, and six months during the growing season, all showed some browning, either locally or all over the seeds; and in all there was some softening of the fibers as indicated by easy disintegration with even careful washing to remove soil particles. Microscopic examination showed the presence of fungi in all cases, often in quantity. That these were different in form and size from those occurring in seeds in the peat medium was not surprising. Inasmuch as no fungi could be located in the ovary, ovule, or developing fruit at any stage, and the progress of the fungus is from the surface inward, it seems clear that infection must come from the medium, which would support different fungi in the individual cases. This will be considered further in relation to sterilization experiments.

Examination of seeds was usually made on those which had been freed from the fleshy pericarp before being placed in the moist medium. In one series in which all the pericarp was retained for comparison with similar cleaned seeds, the superficial appearance after approximately two and one-half months at 25°C., gave evidence of greater infection in the cleaned seeds. They were in part very dark black-brown, or much mottled and streaked, while those left in the flesh were much lighter, even to light yellow. Obviously the necessity of penetration of the extra fleshy layers of the pericarp by the fungi before their entrance into the fibrous portion, delayed changes in the latter region.

The components of the cell walls of the coat, as indicated above, are lignin, pentosans, and cellulose. These are also found in varying proportions in straw, the decomposition of which has been the subject of numerous investigations (Norman (7), Waksman (9), and others). Sterilized straw, either with pure cultures of fungi or with a mixed soil flora, is attacked within a wide temperature range (20 to 50°C., with a maximum at 30° to 35°C.) when sufficient moisture is available (9). To test whether

moist peat at 25° and 30° C. would fulfill the necessary conditions here and supply the fungi required, rye straw in short pieces was sterilized in the autoclave and kept at 25° or 30° C. in moist peat like that used as a medium for *Symphoricarpos* seeds. Within six weeks, the straw in peat was brown and part of it pliable instead of stiff as at the beginning. Sections showed fungi in various tissues with both hyphae and compact bodies, possibly spores. The cell walls in the straw were now less strongly doubly refractive in polarized light than at first. Sterilized straw in tubes of potato dextrose agar retained the original appearance in large degree, with very little change in color and no evident loss of stiffness or other indication of change of character of walls. In the moist peat, the changes correspond to those in seed coat walls, that is, darkening of color with release of tannins, softening and the difference in polarized light due to loss of wall components, all of which are related to the activity of fungi.

Morphology of fungi. The mycelium most frequently encountered in the seeds kept in moist peat (Fig. 6), was rather coarse, septate, branching, and in older hyphae, brown in color. Fine branches went through the pits into adjacent cells. Especially with longer time, septate swollen bodies developed in the cavities of the fibers. It is believed that these may give rise to the oval brown bodies with thick walls (Fig. 2 C) which are often found in the lumen of the fiber after a time. These vary in size but 16 to 20 μ and 10 to 12 μ may be considered representative length and width.

In other cases, there were produced more numerous, somewhat spindle-shaped, two-celled bodies, also with thick, highly refractive walls (Fig. 6 S). In one set, these averaged from 10 to 12 μ in length and 4 to 8 μ in width; in a very different series, the measurements were a little higher, 10 to 16 μ by 7 to 10 μ .

Sterilized seeds. Preliminary experiments in which seeds were superficially sterilized by means of calcium hypochlorite (10) for 5 to 30 minutes, or by 0.2 to 0.5 per cent silver nitrate for very short intervals (one second to two minutes), and transferred to potato dextrose agar, showed no superficial development of fungi after two months at 30°C. In whole material, the seeds were still light in color. Those from the calcium hypochlorite set, pickled intact and sectioned for microscopic study, revealed no fungal hyphae in the outermost cell layer, the fibers, or any other tissue. The crystal layer was intact. Under polarized light, the walls of the fibers appeared firm and thick and strongly doubly refractive; the pits appeared as in untreated seeds.

Seeds previously kept in moist peat for 48 days at 30°C. and similarly treated with calcium hypochlorite or silver nitrate solutions, allowed development of much mycelium, especially from the micropylar end. It was therefore concluded that the treatment served to destroy surface infection without killing more deeply living fungi.

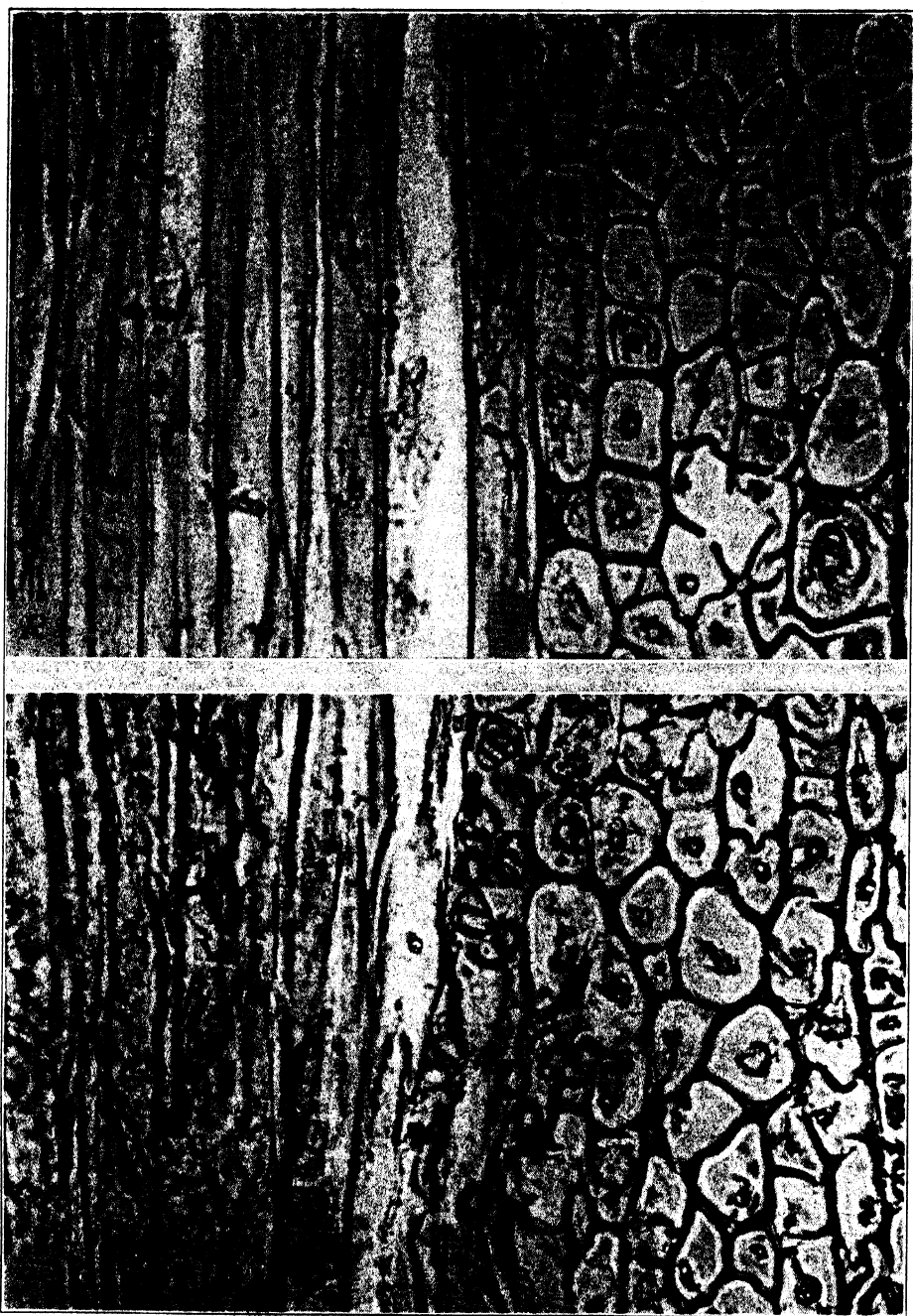


FIGURE 6. Coat of *Symphoricarpos* seeds in moist peat moss. R. Seed at 25° C. three months; f, septate fungus hypha in innermost fiber of outer zone; partly dissolved crystals in adjacent layer. X930. S. Seed at 25° C. four months; two-celled fungus bodies in lumina of long fibers. X930.

Other sterilized seeds studied came from material sterilized in quantity by Flemion who used different technique for this purpose (3). The major part of these seeds were transferred to the peat medium with a low temperature after three months in the sterilized sand at 25° or 30° C. After five months in the moist peat, there was local infection of only two or three outer fibers in most cases; the greatest infection observed involved less than the total depth of the outer zone fibers. Infection was also restricted in longitudinal extension, with a net result of really limited development of fungi.

The coats were hard to cut, and were not colored except as a result of the reagents used in sterilizing. The walls of the coat cells appeared doubly refractive in polarized light when examined at the termination of the interval at 25° or 30°C., and subsequently after periods of approximately seven weeks and three and five months in the low temperature. Microchemical tests showed the presence of lignin, pentosans, cellulose, and the cuticles occurring apparently in the same relations as before treatment of the seeds. Changes in the embryo are not precluded by the lack of change in the coat although the latter apparently results in the failure of germination (3) which would normally follow upon these embryo changes. Measurements of a limited number of embryos indicate a tendency toward increase in size during the cold treatment, as in seeds not kept free from fungi.

Sulphuric acid treatment. Examination of seed coats after treatment with sulphuric acid showed progressively more disintegration of the fibers with longer intervals in the acid, although this is not uniform all over the surface. In an hour and a half, all the outer zone may be destroyed along the sides of the seed, or only two or three of its fibers remain; more may be expected at the ends because of greater original thickness there. In intermediate intervals, as three-quarters of an hour, four or five of the outer zone of fibers may be left, apparently unaffected by the acid. The walls of the fibers not disintegrated appear in polarized light much like those of the control; and the crystals in the middle layer remain unchanged unless the layer is actually destroyed.

Keeping such acid-treated seeds in moist peat at 30° C. soon makes it possible for fungi to gain a foothold. In two weeks, there is but slight infection, and this only in the seeds exposed longest to the acid. In a month, infection is apparent in all acid-treated seeds. In those exposed longest to acid, fungi have penetrated the fibers of both zones and many of the dark brown oval cells have appeared, while the fiber walls have become brown and thin, losing much of their double refraction in polarized light. In seeds exposed to acid for the shortest interval, fungal hyphae occur in only the outermost four or five fibers, much as in the control. The treated seed coats, however, are always thinner so that at any definite

stage a larger proportion of the cells is subject to fungus action than in the control. With little fungus distribution, it is difficult to note any change in polarized light, but with a considerable aggregation of hyphae in adjacent fibers, there is weaker double refraction.

Seeds treated with acid, held in moist peat at 30° C. for two weeks or a month respectively, followed by 5° C. for approximately nine months, showed different percentages of germination (3). In general, those held longer at 30° C. appeared darker and more uniformly brown, except for the controls; in either series, the seeds treated with acid longest showed the greatest change in color and in hardness. That the extreme softness may not be entirely advantageous is indicated by a lower germination rate, which is probably correlated with the action of fungi. These are present in all seeds in both series, with least infection in seeds with least exposure to the acid-higher temperature combination, and most infection at the other end of the series, where fungi sometimes penetrate the old integument parenchyma and even the endosperm. In such cases, where the fungus dominates, it is clear that the treatment is too much in its favor. That such invasion is more apt to occur at the micropylar end is probably related to development of fungi in the placenta region as well as chemical change of the cuticle by the acid in this region.

Comparison of a limited number of embryos in the series treated with acid shows slight increase in size after the acid, possibly not of significance. After two weeks at 30° C., there seems to be but little change in those measured. Subsequent keeping at 5° C. brings about an appreciable increase in length; of those measured, the average proved larger in the seeds held for two weeks at 30° C. before keeping at 5° C., than in those with no exposure to the higher temperature. There is greater differentiation in the larger embryos with beginning of activity in the region of the stem tip. Control embryos showed least increase in size or differentiation. This interpretation is based on averages, since the original material shows wide variation in size of embryos. It is not conclusive because of the small numbers used.

PERMEABILITY TESTS

By far the larger portion of the seed is covered by more resistant tissue in which, however, one small break occurs which is filled with thin-walled tissue (Fig. 1). This is in the region of the original placenta, where the fibrovascular bundle from the ovary vascular supply passes through the funiculus (Figs. 4 H and 5 K) into the old integument tissue and almost encircles the endosperm, either with no branches or very short lateral ones. On the basis of entrance of solutes it appears that this restricted thin-walled tissue in the placenta region allows even more rapid entrance of water than the fibrous cells. Seeds soaked for various intervals from

30 minutes to 2.5 hours, in solutions of copper sulphate (2.5 to 5 per cent), then washed and dried of excess water, and soaked in solutions of potassium ferrocyanide for equal periods of time, washed and sectioned on the freezing microtome, showed the red-brown precipitate from the interaction of the salts in the outermost four fibers, in the thin-walled cells, and along the crushed integument parenchyma for some distance from the placenta. The deposition was especially marked along the fibrovascular bundle of the integument, where it could be seen even at the extreme chalazal end. In longer exposures, the precipitate could be seen in deeper layers of fibers. Similarly, seeds soaked in dilute solutions of methylene blue were colored in half an hour in the outermost fibers, in the placenta tissue and in the fibrovascular bundle of the integument near the micropylar end. In longer standing in the solution (two days), there was blue all through the fibers although more striking in the outermost; the color was variable in amount in the integument epidermis, sometimes faint but sometimes intense; it was visible in the integument parenchyma and always striking in the micropylar region in the fibrovascular bundle and sometimes in the chalazal region. It seems clear then that there is relatively easy entrance of water both into the coat fibers and through the thin-walled tissue near the old placenta.

The strongly developed inner cuticle between the endosperm and old integument tissue apparently does not allow the easy entrance of water. In the tests with salts and methylene blue described above, there was no evidence of entrance of any of the solutes into the endosperm in the time intervals used. It is possible that there may be slow movement of water through this cuticle; it is also possible that changes not readily apparent may make for greater permeability later on. The thin outer cuticle between the fibers and the integument epidermis is of less importance with regard to entrance of water since it is not continuous in the micropylar region.

DISCUSSION

According to previous investigators except Vidal (8), the tissues of the *Symphoricarpos* seed are derived from the ovule. Vidal, whose work on *Sambucus* is substantiated by that of Farmer (2), believes this genus and *Symphoricarpos* similar in the formation of a drupe, of which the stone comes from three inner layers of the ovary wall. The present work confirms his findings on *Symphoricarpos* as far as the general development of the ovary wall is concerned. In comparison with the detailed results of Farmer in the ovary wall of *Sambucus nigra*, the inner zone of fibers appears to originate similarly except that *Symphoricarpos* shows, in addition to the overlapping of already existing cells, some tangential divisions which Farmer reports not to occur in the elder; the outer zone of fibers in *Symphoricarpos* appears to be similar to the tissue arising from

next to the innermost layer of the ovary wall by divisions parallel with the long axis of the ovary of *Sambucus*. There is no development of cells in *Symphoricarpos* comparable to the outermost prismatic layer described in *Sambucus*.

The present work describes in addition the contribution of the epidermis of the integument with crushed parenchyma layers, and the two cuticles (Figs. 4 and 5). Contrary to Netolitzky's finding (6), the outer, rather than the inner, epidermis of the integument is retained in the mature seed. The impermeability of the inner cuticle is considered to have a bearing on germination as the resistance of both cuticles to the progress of fungi has on the well-being of the seed.

The decomposition of the fibers of the seed coat (i.e., the indehiscent stony layer of the drupe) by fungi (Fig. 2) or other agent appears to be a necessary precursor of germination, inasmuch as seeds kept free from fungi fail to germinate. Since the fibers allow the entrance of water and the outer cuticle is interrupted at the micropyle, this appears to be less a matter of permeability than of mechanical difficulty due to the heavy resistant walls of lignin, pentosans, and cellulose.

Examination of a limited number of embryos seems to indicate slight increase in size after treatment of seeds with sulphuric acid or after keeping at 30° C. in a moist medium. Greater change in size and differentiation occurs at 5° C. (Fig. 5 P), especially if this is preceded by treatment with acid or exposure to 30° C. with fungi present, or a combination of acid and fungus action. It would seem that there is some interrelation of coat change and embryo change, although the latter is not entirely dependent on the former.

No attempt has been made in the present investigation to extend the results obtained in regard to coat changes by fungi to other seeds than *Symphoricarpos*. But it is deemed entirely possible that fungi may play a rôle in the disintegration of hard layers of other seeds or fruits, especially indehiscent forms, comparable to that described here.

SUMMARY

In *Symphoricarpos racemosus* the mature seed coat (in less restricted sense) is derived chiefly from the innermost layers of the ovary walls, associated with a little tissue from the integument.

The inner epidermis of the ovary wall gives rise to a narrow zone of pitted heavy-walled fibers, the adjacent cells develop a crystal-bearing layer, and the next few layers a wide zone of fibers similar to the inner ones, but oriented at right angles to them. A single less modified layer of cells covers the fibers.

The integument tissues are represented by a strongly thickened outer

epidermis and a number of layers of crushed parenchyma cells. No nucellus cells remain.

There are two cuticle layers, one between the fibers and the integument epidermis, the other thicker one between the crushed integument parenchyma and the endosperm.

The components of the fiber walls and the integument epidermis include cellulose, pentosans, and lignin. Deposition of the substances is in this sequence; the greater deposit of lignin is in the integument epidermis.

These wall substances become subject to decomposition by fungi from the medium when seeds are kept in moist peat moss or soil at favorable temperatures. The coats soften and readily disintegrate, thus removing a mechanical barrier in seed germination.

The coats of seeds kept free from fungi, but under similar conditions of moisture and temperature, do not undergo these changes.

In seed coats exposed to sulphuric acid for different periods of time, the fiber tissue is reduced in amount in proportion to the length of exposure. The longer exposures favor the development of fungi in subsequent holding in peat moss at 30° C. Too long exposure is disadvantageous for seed germination probably because of excessive development of fungi, possibly because of change in the inner cuticle rendering it non-resistant to fungus entrance.

The fibers of the coat and the thin-walled tissue in the placenta region are permeable to water, as indicated by entrance of salts and methylene blue. The inner cuticle is apparently impermeable. Both inner and outer cuticles seem to be barriers to the progress of fungi under normal conditions.

The embryo is small with a short suspensor, radicle, short hypocotyl and cotyledons and an undeveloped stem tip. There seems to be a tendency toward increase in size and differentiation with keeping in moist peat moss at 5° C., which is more marked if this is subsequent to exposure to sulphuric acid and an interval at 30° C.

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TIME RELATIONS IN EFFECT OF ETHYLENE CHLORHYDRIN IN INCREASING AND OF ETHYL ALCOHOL IN DECREASING THE RESPIRATION OF POTATO TUBERS¹

LAWRENCE P. MILLER

Previous work has shown that when potato (*Solanum tuberosum* L.) tubers are subjected to the vapors of ethylene chlorhydrin (2, 3) or ethyl alcohol (2) marked changes in the respiratory activity as measured by the CO₂ output take place. In these experiments the tubers were exposed to the chemicals in closed containers for a period of 24 hours after which a stream of CO₂-free air was drawn through the containers and through Van Slyke-Cullen (4) tubes containing standard solutions of Ba(OH)₂ to absorb the CO₂ given off by the tubers. The first figure obtained for the CO₂ output thus included the CO₂ given off by the tubers during the 24-hour treatment period and for a number of hours thereafter. These values showed large increases in the case of chlorhydrin treatments and large decreases in the case of treatments with ethyl alcohol indicating that the effects of these chemicals on respiration began rather soon after the start of the treatments.

In the work reported in the present paper experiments were conducted to find out how long a period of exposure to the vapors of these chemicals was necessary until a change in the CO₂ output could be demonstrated. It was found that for ethylene chlorhydrin a definite increase in the respiratory rate begins about twelve hours after the start of treatment and that a decrease in the respiration in the presence of ethyl alcohol takes place within about two hours.

The effect of subjecting potatoes to various combinations of chlorhydrin and ethyl alcohol treatments was also investigated.

METHODS

The method used for the determination of the CO₂ given off was the same as that described previously (2). It was necessary, however, to modify the method of treatment since it was desired to determine the CO₂ at short intervals during the time the tubers were exposed to the chemicals. Instead of placing a definite amount of the chemical in closed containers with the potato tubers for 24 hours, a stream of air carrying the vapors of the chemical studied was drawn through the desiccator containing the

tubers. By interrupting the stream of air for a minute or two it was possible to change the $\text{Ba}(\text{OH})_2$ tubes at any time desired and to determine the total CO_2 given off during the period in question. Vapors of the chemical under consideration were supplied to the stream of air by drawing the air through three Van Slyke-Cullen tubes each containing 50 cc. of the chemical. The solutions used were the 40 per cent solution in the case of chlorhydrin treatments and 95 per cent ethyl alcohol for the alcohol treatments. Control tubers were handled the same way except that they were not exposed to the vapors of the chemical. The air was drawn through the system at the rate of about 14 liters per hour. The temperature throughout the experiments was kept constant at 26°C . by a thermostatically controlled water bath.

Since ethylene chlorhydrin slowly undergoes hydrolysis (1) resulting in the formation of hydrochloric acid it was necessary to take precautions so that none of the hydrochloric acid thus produced would be permitted to react with the $\text{Ba}(\text{OH})_2$ and be computed erroneously as carbon dioxide. In the case of chlorhydrin treatments the air leaving the desiccators containing the tubers which were being treated was therefore first drawn through two Van Slyke-Cullen tubes each containing 25 cc. of 10 per cent silver nitrate solution to remove hydrochloric acid and chloride before it was drawn through the tubes containing the barium hydroxide solutions. A small amount of ethylene chlorhydrin passed through the silver nitrate solution unaltered and was hydrolyzed in the barium hydroxide solutions with the formation of barium chloride. A correction for this was made by determining the chloride content of an aliquot of the barium hydroxide solution by adding an excess of standard silver nitrate solution and titrating back with potassium thiocyanate. The corrections necessary were always small.

RESULTS

With ethylene chlorhydrin. The results with ethylene chlorhydrin treatments are given in Table I. The first two lots show no appreciable increase in respiration up to 11.8 and 9.1 hours respectively but large increases within the next 12 hours, while in the third experiment an increase is shown somewhat before 12 hours (since the value given for the rate from 0 to 12.6 hours is the average rate for the whole period, during most of which the rate of both samples was the same, the actual rate at 12 hours was no doubt several times that of the check). In the fourth experiment an increase is probably present at 12 hours and certainly very soon after 12 hours. On the basis of these data and a number of other experiments not shown it is concluded that the time required for an ethylene chlorhydrin treatment to double the rate of CO_2 output is about 12 hours, with a variation in different lots of tubers of a few hours in either direction.

TABLE I

TIME RELATIONS IN EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON THE RESPIRATION OF POTATO TUBERS

Duration of treatment, hrs.	Hours from start of treatment	Mg. CO ₂ /100 g./hr.	
		Tr.	Ck.
24.0	0 - 2.2	1.05	0.89
	2.2- 5.0	1.10	1.01
	5.0- 7.2	1.19	1.02
	7.2-11.8	1.29	1.15
	11.8-23.3	2.98	1.24
	23.3-27.7	6.52	0.98
21.7	0 - 9.1	2.24	2.11
	9.1-21.7	9.52	2.52
23.1	0 -12.6	3.66	2.00
	12.6-23.1	11.50	2.06
24.0	0 -12.3	1.36	1.05
	12.3-16.3	2.87	1.12
	16.3-19.8	4.25	1.42
5.5	0 - 5.5	1.24	1.13
	5.5-23.3	1.97	1.36
	23.3-29.5	3.29	1.40
5.0	0 -22.0	2.20	1.29
	22.0-48.0	2.90	1.27
5.0	0 - 5.0	1.68	1.34
	5.0-22.0	5.18	1.37

Such a time interval is necessary because of at least two factors; the length of time necessary for a sufficient amount of chlorhydrin to be absorbed by the tubers, and the time required for the absorbed chlorhydrin to influence the CO₂ output. That it is not merely a question of the rate of absorption of the chlorhydrin is shown by the last three treatments given in Table I. It is seen that an exposure of five hours is sufficient to more than double the CO₂ output provided enough time is allowed to elapse to permit the chlorhydrin to affect the respiration.

With ethyl alcohol. The time required for ethyl alcohol to depress the respiration of potato tubers is shown in Table II. With whole tubers a depression in the CO₂ output can be demonstrated about two hours after the start of treatment. The table also shows that it is possible to depress the respiration of cut pieces of potato tubers, which respire at a very high rate, and that the depression in this case also becomes evident within a few hours after the start of the treatment.

With combinations of ethyl alcohol and ethylene chlorhydrin. Since these two chemicals are so active in influencing respiration in opposite directions it is of interest to determine the effect of various combinations of alcohol

TABLE II
TIME RELATIONS IN EFFECT OF ETHYL ALCOHOL ON THE RESPIRATION OF POTATO TUBERS

Whole tubers or cut pieces	Hours from start of treatment	Mg. CO ₂ /100 g./hr.	
		Tr.	Ck.
Whole tubers	0 - 3	0.38	0.95
	3 - 6	0.10	1.26
	Before treatment	1.16	1.13
	0 - 2	0.89	1.36
	2 - 4	0.24	1.27
	4 - 6	0.14	1.57
Cut pieces	0 - 2.3	4.06	4.63
	2.3- 6.7	3.79	8.07
	6.7-18.7	5.42	16.20

and chlorhydrin. Experiments on this point have shown that alcohol can depress the respiration of tubers greatly stimulated by chlorhydrin and chlorhydrin can increase the respiration of tubers the respiratory activity of which has been greatly depressed by alcohol. Such results are given in the first two treatments shown in Table III. Thus in the case where the tubers were first treated with chlorhydrin the treated tubers gave off about twice as much CO₂ as the controls during the first 24 hours. If not treated further the respiration would have increased still more during the next 24 hours (2) but by treatment with alcohol not only was a further increase prevented but the respiratory rate was depressed below that of the first 24-hour period. The data of the second test listed in the table show an increase resulting from a chlorhydrin treatment of tubers which had been depressed over 50 per cent by alcohol vapor.

From a knowledge of the difference in the time relations in the effect of these two chemicals one would expect it possible to treat tubers with chlorhydrin for a length of time sufficient to result in an eventual increase in respiration, but to prevent this increase by treating with ethyl alcohol before the increased CO₂ production due to the chlorhydrin treatment becomes evident. Such an experiment is shown in the third treatment listed in Table III. From the data in Table I it is seen that a five-hour chlorhydrin treatment will bring about a large increase in CO₂ output a number of hours after the end of the treatment. In the present experiment a five-hour treatment period was immediately followed by an alcohol treatment and the depressing action of the alcohol took effect before the chlorhydrin had had time to cause an increase.

The last two experiments given in Table III permit a comparison between a treatment made with chlorhydrin dissolved in ethyl alcohol and a treatment with the same amount of chlorhydrin dissolved in water. These treatments were made by exposing the tubers in a closed container

to the vapor from 1 cc. of the solutions per liter of air space for 24 hours. While the presence of the alcohol did not entirely prevent an increase in respiration as compared with the control, the increase was much less than that resulting when the same amount of chlorhydrin was used in aqueous solution. It is of interest that although the presence of the alcohol with the

TABLE III

EFFECT OF VARIOUS COMBINATIONS OF ETHYLENE CHLORHYDRIN AND ETHYL ALCOHOL TREATMENTS ON THE RESPIRATION OF POTATO TUBERS

Treatment	Hours from start of treatment	Mg. CO ₂ /100 g./hr.	
		Tr.	Ck.
Chlorhydrin 0.6 cc. for 21 hours followed by alcohol 2.0 cc. for 21 hours*	0 -23.7	4.04	2.04
	23.7-46.9	3.06	1.92
	47.3-70.7	3.54	2.04
Alcohol 2 cc. for 21 hours followed by chlorhydrin 0.6 cc. for 21 hours*	0 -23.7	0.93	2.04
	23.7-47.3	2.40	1.92
	47.3-70.7	2.95	2.04
Chlorhydrin for 5 hrs. followed by alcohol for 22.5 hours**	0 - 5.0	1.77	1.53
	5.0-27.5	0.36	1.49
	27.5-52.7	0.66	1.45
	52.7-71.5	1.03	1.29
10% chlorhydrin in 95% alcohol, 1 cc. for 24 hours†	0 -26.0	0.61	0.70
	26.0-43.5	1.46	0.71
	43.5-48.0	1.98	1.06
	48.0-72.0	1.96	0.88
10% chlorhydrin in water, 1 cc. for 24 hours†	0 -26.0	1.12	0.70
	26.0-43.5	4.58	0.71
	43.5-48.0	5.34	1.06
	48.0-72.0	2.04	0.88

* These treatments were made by subjecting the tubers in closed containers to the vapor from a definite amount of the chemical placed on cotton in the container. The concentrations are expressed as cc. of 40% chlorhydrin or 95% alcohol per liter of air space in the container.

** The treatments in this experiment were made by passing the vapor of the chemicals over the tubers as described earlier in the paper.

† These solutions were made by adding 10 cc. of 100 per cent ethylene chlorhydrin to 90 cc. of alcohol and water, respectively.

chlorhydrin reduces considerably the respiratory increase which results from a chlorhydrin treatment, a number of comparisons of the two solutions (i.e., 10 cc. chlorhydrin plus 90 cc. 95 per cent alcohol and 10 cc. chlorhydrin plus 90 cc. water) with regard to their action on dormancy has shown that the efficacy in breaking dormancy is not reduced by the presence of the alcohol.

SUMMARY

The increase in respiration (CO₂ output) which results when potato tubers are exposed to ethylene chlorhydrin vapor begins about 12 hours

after the start of treatment. The decrease resulting from exposure to ethyl alcohol occurs in about two hours from the start of treatment.

The respiration of tubers depressed by ethyl alcohol can be stimulated by ethylene chlorhydrin treatments and the stimulation of respiration resulting from ethylene chlorhydrin treatments can be reduced by treatments with ethyl alcohol. These two chemicals also tend to counteract each other when potato tubers are subjected to the vapors of both of them at the same time.

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IMPROVEMENTS IN METHODS OF DETERMINING STARCH IN PLANT TISSUES

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The older methods for the analysis of starch were based upon the fact that starch, or the products of the enzymic hydrolysis of starch, such as dextrin and maltose, were hydrolyzed to dextrose by heating with dilute acid, and that from the amount of dextrose so formed the amount of starch could be calculated.

Subsequently the methods that involved acid hydrolysis even after a preliminary enzyme digestion were criticized from two points of view (a) that the results were too high because accompanying non-starch polysaccharides were hydrolyzed and were estimated as starch; (b) that the results were too low because of a destruction of dextrose (and possibly maltose) by acid in the process of acid hydrolysis.

In the more recently developed methods for the determination of starch in plant tissues, acid hydrolysis has been omitted at all stages. Instead takadiastase is used, and the calculations are based upon the assumption that only starch has been hydrolyzed by the enzyme, and that the only products formed from the starch are maltose and dextrose, from the amounts of which the starch originally present could be determined by any one of at least three methods (5, 18, 21).

When dealing with tissues low in starch but high in accompanying non-starch polysaccharides, as is often the case in experiments in plant physiology, the difficulties in getting a true measure of the starch become accentuated. In attempting to apply different starch methods to plant tissues, many of which were low in starch, and some of which were starch-free, the following questions arose: Are glucose and other hydrolytic products of starch destroyed by heating with acid under the conditions usually recommended for starch hydrolysis? What are the products formed in the hydrolysis of starch by takadiastase? Is takadiastase specific for starch, and may the increase in copper-reducing power resulting from the contact of takadiastase with the tissue be regarded as having resulted only from the hydrolysis of starch? Can the starch which is present in plant tissue enclosed within the cells be completely removed from plant tissue, so that the residue no longer gives a test for starch?

As a result of these tests with tissue from 12 different species, including leaves, stems, tubers, roots, fruits, and seeds, partial or complete answers to these questions were obtained, and there were developed for starch in plant tissues two different procedures, the details of which are described

together with the results obtained with them in comparison with those by other well-recognized methods.

MATERIALS AND METHODS

Species used. The species used in these experiments were: salvia, *Salvia splendens* Ker.; cotton, *Gossypium hirsutum* L.; grape, *Vitis labruscana* Bailey; apple, *Pyrus malus* L.; lilac, *Syringa vulgaris* L.; orange, *Citrus sinensis* Osbeck; cantaloup, *Cucumis melo* L.; potato, *Solanum tuberosum* L.; celery, *Apium graveolens* L. var. *dulce* DC.; soybean, *Glycine max* Merr.; dahlia, *Dahlia variabilis* Desf.; gladiolus, *Gladiolus* L. var. Alice Tiplady.

Preparation of tissues. The tissues were cut with knife or scissors into small pieces which were dropped into boiling 95 per cent alcohol to give a final concentration of about 80 per cent alcohol. The insoluble material was collected in Soxhlet extraction thimbles and was extracted with 95 per cent alcohol for about 40 hours. The residue was placed in beakers and was covered with an excess of ethyl ether and allowed to stand for 1 hour, after which the ether was decanted and discarded. The tissue was extracted with ether four times in succession in this way. Finally the tissue was placed on an evaporating dish until dry, and was ground in a food grinder and with mortar and pestle until the tissue powder passed through a 60-mesh sieve. Calculations were based on this air-dry powder.

Sugar determinations. For sugar solutions containing more than about 15 mg. of glucose per 100 cc. the Munson and Walker method (1, p. 379) was used and the cuprous oxide was titrated with 0.05 N KMnO_4 . With tissues containing small amounts of starch, however, the final solution contained amounts of glucose too small to permit the use of this method, and in such cases a modification involving both the Shaffer and Somogyi (17) and Harding and Downs (8) methods was used. The principal modifications consisted in using a larger volume of the sugar solution and in separating the cuprous oxide from the copper-sugar mixture before the iodate-iodide solution was added, the object being to avoid error due to non-sugar iodine-absorbing substances in the liquid obtained by digesting the plant tissue with *takadiastase*. The solutions used were: (A) containing 15 g. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per l.; (B) containing 50 g. Na_2CO_3 , 40 g. NaHCO_3 , and 50 g. Rochelle salt ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) per l.; (C) containing 10 g. KI and 0.8 g. KIO_3 per l.; (D) containing 180 cc. of conc. H_2SO_4 per l.; (E) containing 36 g. of potassium oxalate per l. Equal portions of solutions A and B were mixed just previous to their use and 25 cc. of the mixed solution were added to 25 cc. of the sugar solution in a large test tube. This was heated for 15 minutes in a bath of vigorously boiling water under conditions similar to those described by Shaffer and Somogyi. Several tubes were usually heated simultaneously. The tubes were cooled in running

water, were centrifuged, and the liquid above the cuprous oxide was decanted. Then 10 cc. of solution C were added with a pipette and immediately 10 cc. of solution D, and 10 cc. of solution E. The mixture was allowed to stand for 5 minutes and was then titrated with 0.01 N sodium thiosulphate. The procedure was standardized against a sugar solution made from pure dextrose obtained from the U. S. Bureau of Standards. Amounts as low as 0.5 mg. per 25 cc. could be estimated by this method, and with amounts up to 4 mg. per 25 cc. the corresponding thiosulphate values plotted approximately on a straight line.

Starch determinations. The details of the starch methods are described in subsequent paragraphs in the description of the experiments on the analysis of different plant tissues for starch content.

EFFECT OF HCl UPON GLUCOSE, MALTOSE, AND STARCH

Judging by recent articles (18, 19, 22) the generally accepted view is that a destruction of glucose occurs under the conditions for acid hydrolysis of starch. The evidence for this conclusion, however, is not convincing. The fact that low results were obtained when the sugar-containing solution was heated with acid does not justify the conclusion that glucose was destroyed or that acid caused its destruction. The previous work of Davis and Daish (4) is usually cited but an examination of their data shows that the evidence for glucose destruction is not convincing. Only one test was made with an acid concentration and time of heating similar to that which prevails in the hydrolysis of starch (4, p. 456, Table V). Of the original 0.8216 g. starch they found 0.8140 g. after hydrolysis, which is a calculated loss of 0.0076 g. This is less than 1 per cent of the amount taken, and it may be doubted whether any loss is shown. Destruction of dextrose was proved by Davis and Daish only when they used a higher concentration of HCl for a longer period of heating.

Curiously enough the work of Noyes et al. (14) is often cited as evidence of the destructive action of acid upon glucose; but the conclusion of these authors on this point was: "the *reducing* power of the glucose solution, as determined by Fehling's solution, is not appreciably altered by heating with acid" (14, p. 269). It is true that they reported a decrease in the reducing power of *malto*se when the heating with acid was continued for periods longer than one hour. Their data, however, do not entirely confirm this view. Adopting their suggestion that the true measure for comparison is the per cent of copper oxide rather than the per cent of hydrolysis, we turn our attention to their Series I, II, III, IV (14, p. 271, 272) and find 15 independent comparisons between the per cent of total copper oxide for periods of heating of one hour or longer. Comparing all possible pairs such as one hour against two, one against four, one against six, two against four, etc., we find that in seven cases the cuprous oxide

values are lower for longer periods of heating, and in eight cases they are higher, furnishing no conclusive evidence that longer heating with acid reduces the amount of reducing sugar.

Decrease in copper-reducing power as a result of heating plant tissue or plant tissue extracts with acid cannot be used to prove destruction of glucose by acids when such tissues or tissue extracts contain *levulose* or substances which give levulose upon hydrolysis. It is well known and easily demonstrated that levulose is destroyed by heating with acid under the conditions for starch hydrolysis.

In view of these differences of opinion regarding the effect of heating glucose or other hydrolytic products of starch with acid the following experiments were carried out to furnish additional evidence on the subject. The point of view has been not to attempt to determine whether all of the glucose, maltose, or starch originally taken could be accounted for in the liquid at the end of the period of heating with acid, but rather to attempt to find what concentration of acid is necessary to produce an unmistakable destruction of glucose or other substances obtainable from the action of acid or amylase upon starch.

To 50 cc. aliquots of a glucose solution, containing approximately 0.2 g. of glucose, varying amounts of concentrated HCl sp. gr. 1.19 were added together with H₂O to make the final volume equal to 110 cc. This mixture was heated in boiling water for 2.5 hours. After neutralization the reducing power of aliquots of this solution was determined and the values in Table I, column 2, show the potassium permanganate titration values of the cuprous oxide precipitates. No significant change in the copper-reducing value of the solution was obtained until the amount of HCl added was about 27 cc., which is about four times the concentration of acid used in the hydrolysis of starch (1, p. 284). In column 3, Table I, are shown the results of a similar test with a solution of maltose. Again about 27 cc. of concentrated HCl per 110 cc. of final mixture are needed to cause a lowering of reducing power. For the test with potato starch the starch paste was digested first with saliva for 16 hours and aliquots of the liquid after digestion were taken for acid hydrolysis. The results, shown in column 4, Table I, are similar to those obtained with glucose and maltose, and show that the concentration of HCl can be varied from 7 cc. to at least 14 cc. and probably much higher without any evidence of destruction of copper-reducing substance in glucose or maltose solutions, or in a solution containing the products formed from the hydrolysis of potato starch with saliva.

Although these tests and a consideration of the data of previous workers do not indicate that acid hydrolysis brings about a lowering of the maximum reducing power obtainable from a given quantity of starch, it is clear that acid hydrolysis should be avoided not because the results may be too low but because they are quite likely to be too high with plant tis-

TABLE I

EFFECT OF THE CONCENTRATION OF HCl UPON THE COPPER-REDUCING CAPACITY OF GLUCOSE, MALTOSE, AND THE ENZYME DIGESTION PRODUCTS OF POTATO STARCH

Cc. of conc. HCl sp. gr. 1.19	Cc. of KMnO_4		
	Glucose soln.	Maltose soln.	Starch after digestion with saliva
0	32.3	17.2	12.7
2	32.0	30.6	28.0
7	32.0	31.3	30.9
7	31.9	30.9	31.4
10	32.2	31.7	31.4
10	32.1	31.3	31.3
14	32.0	30.8	31.1
14	32.2	30.8	30.9
27	30.0	27.4	29.9
34	27.7	21.6	28.0

sues or plant tissue extracts. This results from the hydrolysis of non-starch substances into products with copper-reducing capacity. Consequently correct results for starch are to be expected from methods which avoid the use of acid. In the paragraphs which follow are shown the results of tests of methods by which the starch content of tissues may be estimated without the use of acid hydrolysis at any stage of the process.

EFFECT OF TAKADIASTASE UPON POTATO STARCH

According to the view commonly held at the present time the only products formed by the hydrolysis of starch by takadiastase are maltose and glucose. The experiments of Davis and Daish (5) were of great influence in establishing this view. They found an arrest-point was reached when about 84 per cent of the starch was present as glucose, the remainder being maltose. Subsequently Thomas (19) found the ratio of glucose to maltose to be so nearly 2.0 that calculations made on the basis of that proportion gave dependable measures of the starch that had been hydrolyzed. Shriner (18) makes use of this constant value in an improved method for starch. However, Widdowson (22) did not obtain a constant value for the ratio of glucose to maltose; the value varying in different experiments from about 5.0 to about 2.7.

In contrast to these results which show that the reaction stops before all of the maltose is split to glucose, we find at least four authors who reported a complete hydrolysis of starch to glucose by the use of takadiastase. Collins (3) found in 1927 that if the takadiastase was buffered at pH 5.0, and if a sufficient quantity of takadiastase was used in proportion to the starch present, 98.5 to 101.1 per cent of the starch was accounted for as glucose. Lehmann (11) also reported in 1932 practically complete conversion of starch to glucose by takadiastase but emphasizes the im-

portance of boiling the starch for 40 minutes before adding the takadiastase. But even previous to the reports of Collins and of Lehmann there was evidence in the literature that takadiastase hydrolyzed starch to glucose and not to a mixture of glucose and maltose. Thus, we may interpret from the data given in an article by Philoche (15) in 1908 on measurements of the action of takadiastase upon maltose that the equilibrium which was reached depended upon the quantity of takadiastase taken. Low amounts of takadiastase acting for short periods gave low amounts of glucose but as the proportion of takadiastase and the time of contact were increased the proportion of the maltose which was split to glucose increased until all of the maltose was converted into glucose. For example, he showed (15, p. 247) that, with 2 per cent maltose and takadiastase to the amount of 1 per cent, maltose was converted completely to glucose in 480 minutes. Other convincing evidence is given also in many of the tables from pages 254 to 261 of Philoche's article.

The earliest evidence that the writer has been able to find which indicates hydrolysis of starch completely to glucose is that of Hill (9) in 1901. He said "The hydrolysis of dilute starch solutions by taka-diastase ends in an almost complete transformation to glucose, as shown by the combined polarimetric and copper reduction methods of estimation; no dextrin which resists further hydrolysis is formed and there is sufficient maltase in commercial taka-diastase to convert all the maltose to glucose."

The results of these authors showing complete hydrolysis of starch to glucose have not received the attention they have deserved from subsequent investigators. Possibly the claims of Hill were not entirely convincing since he presented no details regarding the experimental procedure. Also, Philoche did not refer directly to the problem discussed here and the bearing of his results upon it could be obtained only by an examination of the tabular matter. But for overlooking the results of Collins (3) subsequent investigators cannot be so readily pardoned. This author stated clearly the conditions under which these results could be obtained.

If the claims made by these investigators are true the determination of starch in plant tissue should be greatly facilitated, since only glucose should be produced in the hydrolysis of starch and only a single sugar determination would be required from the results of which the amount of starch could be computed at once.

Tests were made in the present experiments on the copper-reducing power of potato starch suspensions to which varying amounts of takadiastase were added. The mixtures of gelatinized potato starch were buffered at pH 4.5 with 0.2 M acetic acid-sodium acetate solutions. At the end of a period of incubation at 38° C. the solution was made up to volume and two equal aliquots were taken. To one of these HCl (sp. gr. 1.125) was added and acid hydrolysis was carried out under the conditions for acid hydroly-

sis of starch (1, p. 282). To the other no acid was added. After the acid hydrolysis was ended the solutions were neutralized and were made up to the same volume. Aliquots were taken for the sugar determination. If the KMnO_4 values were the same without hydrolysis as they were with hydrolysis it was concluded that the takadiastase had transformed the starch completely into glucose, or at least into reducing substances that were not capable of further hydrolysis. These experiments completely substantiated the results reported by Collins (3). When the weight of takadiastase was at least one-half of the weight of starch the hydrolysis was complete, provided the period of digestion was as long as 44 hours. In 24 hours it was not quite complete. The ratio of takadiastase to starch could be reduced to about 1:4 if the digestion was continued for one week.

These amounts of takadiastase are larger than those usually taken for the hydrolysis of starch, and since the samples of takadiastase that were used in these experiments contained reducing substances, high values for the correction for blank were needed in the experiments with plant tissues containing starch. It was found, however, that the takadiastase solutions which were obtained upon dialysis were free of reducing substances, and yet were high in amylolytic power. The collodion bags for this purpose were prepared from a solution of 5 grams of Union Cotton Negative in 50 cc. of ethyl ether and 50 cc. of 95 per cent ethyl alcohol. Usually the takadiastase solutions were dialyzed overnight in running tap water. The volume of solution nearly doubled during the process, but a notation was made of the volume which was reached and in subsequent experiments the amount of dialyzed takadiastase solution was adjusted to give any desired amount of takadiastase in the original solution before dialysis.

The ability of this dialyzed solution of takadiastase to hydrolyze potato starch is shown in Table II. Column 1 shows the relative weights of air-dry potato starch and takadiastase. To 150 cc. of the starch paste, which had been heated to boiling and then cooled, containing 0.375 g. of air-dry starch, 37.5 cc. of a solution containing the proper amount of takadiastase were added, together with 25 cc. of 0.2 M acetic acid-acetate buffer at pH 4.5. With a high proportion of takadiastase to starch the solutions became clear almost instantly. The flasks were placed in an electric oven regulated at 38° C. for 44 hours. Then the solutions were neutralized and made up to a volume of 250 cc. From this 100 cc. were pipetted out, 10 cc. of HCl (sp. gr. 1.125) were added, and this sample was hydrolyzed under the conditions for hydrolysis of starch (1, p. 282), after which it was neutralized and made up to 200 cc. Another 100 cc. aliquot of the original solution was pipetted into a 200 cc. flask and was neutralized and made up to 200 cc. Aliquots of these solutions were taken for the sugar determination, the cuprous oxide being titrated with 0.05 N KMnO_4 . The experiment was carried out in triplicate and the KMnO_4 values are shown

in columns 2 and 3 in Table II. The values in column 2 were calculated as percentages of the values in column 3, and column 4 indicates the percentage of the starch which was hydrolyzed to glucose. It is seen that when the ratio of takadiastase to starch was as high as 2:1 all of the starch was converted into glucose; at a ratio of 1:1 the conversion was nearly com-

TABLE II
EFFECT OF DIALYZED TAKADIASTASE UPON POTATO STARCH

Ratio of dialyzed takadiastase to starch (by weight)	Cc. KMnO_4		% hydrolyzed to glucose
	After digestion of starch with takadiastase		
	Before acid hydrolysis	After acid hydrolysis	
2:1	24.0	23.9	100
	24.5	24.6	
	24.0	24.3	
1:1	23.2	23.7	98
	23.7	24.0	
	23.6	24.3	
1:2	22.4	24.2	94
	22.9	24.1	
	22.6	24.0	
1:4	21.5	24.3	90
	21.5	23.6	
	22.0	24.2	

plete; but at ratios of 1:2 and 1:4 higher values were obtained by hydrolyzing the enzyme digests, indicating the presence of some maltose which had not yet been changed to glucose by the enzyme.

This dialyzed solution of takadiastase was found to be stable, and, after standing for several weeks either at room temperature or at 10° or 3° C., retained its capacity to hydrolyze starch completely to glucose.

EFFECT OF DURATION OF BOILING OF STARCH UPON THE HYDROLYTIC CAPACITY OF TAKADIASTASE

Lehmann (11) reported that one of the factors regulating the capacity of takadiastase to hydrolyze starch was the duration of the period of boiling to which the starch was subjected. He stated that complete hydrolysis could be obtained only when the starch paste had been boiled for 40 minutes previous to the addition of the takadiastase.

This point was tested in the present experiments and the results are shown in Table III. The time during which the boiling temperature was maintained was reduced from 45 minutes to 1 minute without any appreciable effect upon the amount of reducing sugar that was formed by the action of takadiastase. For temperatures below 100° C. the water was first

brought to the desired temperature such as 90°, 80°, etc. and the starch (previously moistened with a little cold water) was added to the water. The mixture was stirred thoroughly and placed at once in a current of tap water.

It will be seen from Table III, columns 3 and 4, that not until the water temperature had been reduced to 60° C. was the hydrolysis of the starch

TABLE III
EFFECT OF TEMPERATURE DURING GELATINIZATION OF POTATO STARCH UPON HYDROLYSIS WITH TAKADIASTASE

Treatment of starch		Cc. of KMnO ₄ after digestion with	
Time of boiling, min.	Temp. of H ₂ O	0.2 g. taka.	0.05 g. taka.
45	100° C.	42.4	39.3
7	100° C.	42.3	39.5
1	100° C.	43.9	40.1
—	100° C.	43.4	40.8
o	90° C.	43.8	40.4
o	80° C.	43.2	40.4
o	70° C.	41.8	39.5
o	60° C.	35.9	33.1
o	50° C.	19.7	7.4
o	40° C.	7.1	5.8

seriously interfered with. Starch grains which had been added to water at a temperature of 40° or 50° were attacked to only a small extent by takadiastase.

Samples of the starch pastes from the 40°, 50°, 60°, and 70° lots described above were examined with a microscope and the microphotographs shown in Figure 1 were taken in polarized light. At 70° the starch grains had swollen so much that the outlines of the individual grains could barely be distinguished; at 60° many but not all of the grains had swollen; at 50° and 40°, however, very little swelling of grains had occurred. Figure 1 shows that the change from 60° to 70° represents the critical point in the swelling of the grains, while the data in Table III show that in the hydrolysis experiments the critical point was from 50° to 60°. When the crosses on the starch grains in polarized light are examined it is seen that although practically all of the grains show crosses in the 50° sample, most of the grains in the 60° lot fail to show crosses or give only indistinct crosses. The hydrolysis data, therefore, correlate better with the effect of the temperature upon the crosses than upon the swelling. The most important gain in the hydrolysis of the starch grain occurred when the molecular arrangement was so changed that crosses were no longer formed in polarized light.

TESTS OF DIFFERENT STARCH METHODS WITH PLANT TISSUES

The experiments with takadiastase indicated that a complete hydrolysis of starch could be obtained under the proper conditions, that acid

hydrolysis of the enzyme digest would be unnecessary, and that the starch content could be estimated directly from the reducing-sugar values. This method was applied upon a number of different plant tissues and the results are shown in column 6, Table IV. The details of the takadiastase method are as follows:

Takadiastase method. If the powdered tissue contains no direct reducing substances a weighed sample is placed in a glass mortar and ground thoroughly with a little water. If

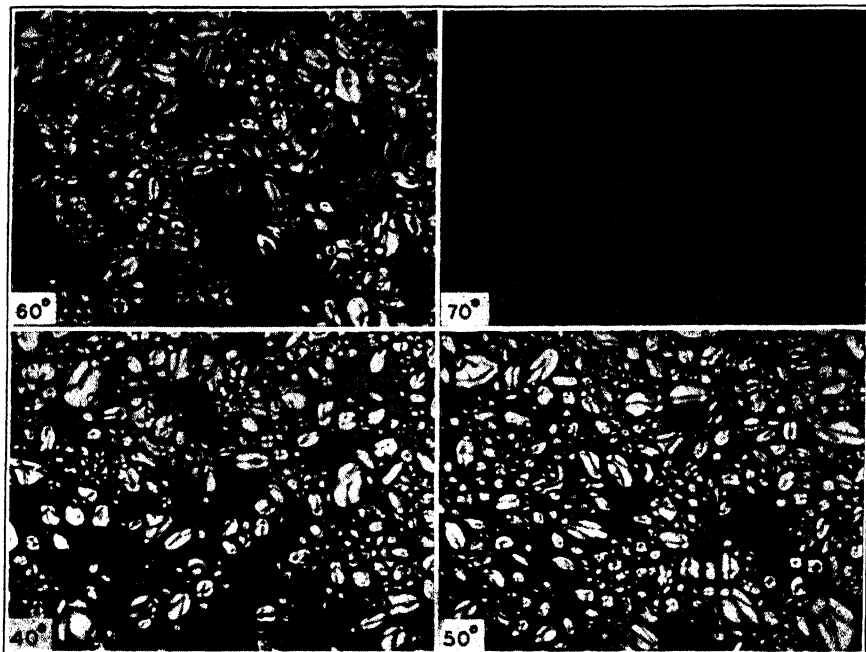


FIGURE 1. The numbers show the temperatures in degrees Centigrade of the water at the instant of adding the potato starch which had been moistened previously with cold water. Photographs made with polarized light. $\times 83$. For the effect of takadiastase upon samples of these starch pastes see Table III.

there are reducing substances present the powder is first put on a filter paper in a funnel and is extracted with 50 per cent alcohol and then with ether. The powder is transferred with a spatula to the mortar, the filter paper being retained upon the funnel until the following day. Thorough grinding of the powder is necessary in order to obtain a negative test for starch on the residue at the end of the experiment. The thoroughly disintegrated powder is transferred to a large test tube with water to make a final volume of about 25 cc. The test tube containing the tissue is placed in a boiling-water bath for one-half hour. After cooling to room temperature, 25 cc. of 0.2 M acetic acid-acetate buffer at pH 4.5 are added and 25 cc. of a takadiastase solution which contains at least as much and preferably twice as much takadiastase as there are grams of starch in the sample. Toluene is added. The test tube is now placed in an incubator at about 38° C. until the following day. (In the

present experiments the tube was stoppered tightly and rotated end over end on a turning bar inside the oven.) The sample is now filtered through the filter paper that has been retained from the previous day into a volumetric flask and a bit of the residue is examined with iodine microscopically for starch. If the test is positive the tissue is again ground with a mortar and pestle and is again returned to the test tube, this time the filter paper itself being added. If the test is negative the filter paper and residue are added to the test tube, and the sample is reincubated with takadiastase and buffer as before. The volumetric flask containing the filtrate is also placed in the incubator. On the following day, approximately 44 hours from the start, the digest is filtered into the volumetric flask, and the residue is washed repeatedly until the liquid in the flask occupies about four-fifths of the volume. Then saturated basic lead acetate solution is added until no further precipitate is obtained. It is made up to volume and delead with dibasic sodium phosphate. Aliquots are taken for the sugar determination and the glucose values are converted into starch by the use of the factor 0.93 (3, 14).

If experience with a given tissue has shown that the residue can be cleared of starch by a single grinding in the mortar the filter paper may be added to the digest at the start of the incubation and the digestion may be continued without interruption until the end of the 44-hour period.

For comparison with the takadiastase method three other methods were employed upon the same tissues. One was a modification of the method first introduced by Walton and Coe (20), later improved by Coe (2), and subsequently adopted by the Association of Official Agricultural Chemists as an official method (1, p. 282). The essential feature of this method is that the interfering polysaccharides are removed from the enzyme digest at the completion of the action of the enzyme by precipitation with alcohol. The acid hydrolysis is then carried out upon an aqueous extract of the alcohol-soluble portion containing the hydrolytic products of starch (dextrin, maltose, and glucose). In the present experiments the method was modified by using saliva at pH 6.5 instead of malt extract and by increasing the time of digestion to 44 hours. The results obtained by this method are shown in column 5, Table IV.

The other method used consisted in modifying the method originally proposed by von Fellenberg (6) and subsequently improved in various ways by himself (7) and by others (12, 13). This method is based upon the solubility of starch in concentrated calcium chloride solution from which the starch is precipitated as starch iodide upon the addition of iodine. The starch in this precipitate has been estimated in various ways by different experimenters but in the present experiments this was done by removing the iodine and calcium chloride from the starch and estimating the latter with takadiastase. The details of the calcium chloride method as finally adopted are as follows:

Calcium chloride method. The weighed powdered tissue is placed in a mortar, is moistened with water, and is disintegrated by grinding. The time used to obtain thoroughness at this stage of the process is well spent. With a fine jet from a wash-bottle transfer the powder to a large centrifuge tube (80 to 100 cc.). In order to know approximately the volume of water used in transferring the powder to the tube the "wash-bottle" used in these experi-

ments was a 100 cc. test tube marked with 10 cc. graduations. Weigh out an amount of crystallized calcium chloride ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$) equal in grams to twice the number of cc. of water used, add it to the tube containing the tissue, place the tube in a boiling-water bath for 20 minutes, remove, bring to a boil over a free flame, and centrifuge, decant the liquid into a liter beaker, and add to it four volumes of water. To the residue in the tube add 20 cc. of concentrated calcium chloride solution (2 parts of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ to 1 part of H_2O , by weight). Again heat in boiling water for a few minutes, boil over a free flame, centrifuge, and decant. After about five successive extractions, the last one of which is preferably an overnight extraction at room temperature, transfer the residue to a filter paper by the use of 95 per cent alcohol and examine a bit of the tissue with a microscope. If the starch test with iodine is positive transfer the tissue to a mortar, grind it, and repeat the extraction with CaCl_2 . To the diluted calcium chloride extracts of the tissue in the beaker add a few drops of a saturated solution of iodine in 10 per cent potassium iodide, and continue adding until there is an excess of iodine. This can be tested by observing the color of the drop of liquid on the end of a stirring rod. Even when the liquid in the beaker is dark blue an excess of iodine will show plainly in the drop of liquid. The color of the bubbles produced by stirring the liquid also gives an indication of the amount of iodine necessary to give an excess. The precipitate of starch-iodide will form within about an hour but until further work is done on this point it will be well to let the beaker stand overnight in order to permit complete precipitation.

The starch-iodide precipitate is transferred quantitatively to an asbestos mat which has been previously prepared in a Büchner funnel using a 60 mm. funnel for small amounts of precipitate and a 75 mm. funnel for large amounts. The precipitate is washed first with cold and finally with hot 95 per cent alcohol. This is to remove the CaCl_2 , the KI, and some of the iodine. With a rubber-tipped rod and some water, transfer the precipitate together with the asbestos to a 200 cc. Erlenmeyer flask, place on a sand bath and boil slowly for a few minutes. The starch-iodide precipitate is broken up by this process and the iodine is volatilized. The flask is then placed on a steam bath and within an hour or two the liquid will be free of iodine as shown by the odor. Cool, add takadiastase and the acetic acid-sodium acetate buffer at pH 4.5, and proceed as shown above for the takadiastase method. If the starch content of the sample is low, as shown by the volume of the starch-iodide precipitate, the volume of the liquid in the takadiastase digest should be kept low so that suitable titration values will be obtained in the sugar determination.

With some of the tissues it was not found possible to obtain a clear liquid when the tubes were centrifuged after heating the tissue with the CaCl_2 solution. If only a few tissue particles were present in the liquid it was not considered that any important error was made by proceeding without reference to them. But when a considerable proportion of the residue was not forced to the bottom of the tube the procedure was modified as follows: the liquid was decanted into a 250 cc. beaker containing four volumes of hot water and was filtered at once through paper into the liter beaker. After several successive extractions were made the residue was transferred to the filter and was examined for the presence of starch. If the test was positive the tissue was transferred to a mortar and ground. An extraction with CaCl_2 was again made.

The results by the calcium chloride method are shown in column 7, Table IV.

Finally each of the tissues was hydrolyzed with acid by the Official method (1, p. 282) and the results are shown in column 4, Table IV. This gives a measure of all of the easily hydrolyzable material whether it is

starch or non-starch and is included only for comparison with the results by the methods more nearly suitable for the determination of starch.

TABLE IV
STARCH CONTENT OF VARIOUS TISSUES BY DIFFERENT METHODS

Plant	Tissue used	Qual. test for starch present (+) or absent (-)	% of air-dry weight by the following methods				% non-starch†
			Acid. hyd. of powder	Modified Walton and Coe	Takadiastase without acid hyd.	CaCl ₂ extraction	
Salvia*	Leaf	+	23.0	16.20	17.20	9.78	43
Salvia**	Leaf	+	7.2	1.78	1.70	0.96	44
Cotton*	Leaf	+	24.6	12.47	12.78	7.99	38
Cotton***	Leaf	+	14.9	3.92	4.36	3.03	30
Soybean	Leaf	+	22.6	7.00	6.02	4.82	20
Gladiolus	Leaf	—	14.8	1.38	1.34	0.00	100
Grape	Twig	+	23.0	5.24	5.01	5.31	0
Lilac	Cortex	+	21.2	2.24	2.54	1.69	33
Lilac	Wood	+	18.7	1.74	1.28	0.88	31
Orange	Fruit rind	—	22.0	1.20	0.64	0.05	92
Apple (York)	Fruit flesh	+	44.0	15.36	13.20	14.02	0
Apple (Sutton)	Fruit flesh	+	34.8	5.73	5.72	5.13	10
Apple (Greening)	Fruit flesh	+	32.6	1.78	1.44	0.67	53
Apple (McIntosh)	Fruit flesh	+	31.6	2.80	2.82	0.23	93
Potato	Tuber	+	73.6	63.60	63.60	66.60	0
Celery	Stem	+	36.0	14.66	13.40	13.50	0
Dahlia	Root	—	55.6	10.96	0.87	0.00	100
Cantaloup	Seed	—	4.8	0.80	0.65	0.00	100

* Leaves sampled in p.m.; ** leaves taken from plants that had been stored in the dark for 48 hours; *** leaves sampled in early morning.

† Difference between the takadiastase and calcium chloride methods expressed as a percentage of the takadiastase values.

The results by these methods upon 12 different tissues are shown in Table IV. With most of the tissues good agreement was obtained between the takadiastase and the Walton and Coe methods. Dahlia root tissue was an exception, the high value by the Walton and Coe method being due no doubt to hydrolysis by acid of inulin which was not precipitated by alcohol. However, it is clear that with certain of the tissues the results were too high by both of these methods. Thus, orange rind, cantaloup seeds, dahlia roots, and gladiolus leaves, although containing no starch as shown by qualitative tests with iodine both upon the tissue and upon calcium chloride extracts of the tissue, gave appreciable "starch" values by these two methods.

The calcium chloride method gave results which were the same or similar to the other two methods only with certain tissues, and especially with grape twig, potato tuber, celery, and with York and Sutton apple. This method had the merit of showing low or zero values with tissues that

showed no qualitative tests for starch. The results were especially favorable with the four apple varieties, York, Sutton, Greening, and McIntosh. When iodine was applied to the cut surface of the flesh of the apples at the time the tissue samples were taken it was evident that the York variety was highest in starch, followed by Sutton, that Greening had starch only in the peripheral region of the fruit, and that only traces of starch remained in the flesh of McIntosh. These results were confirmed by the appearance of thin sections of the tissue stained with iodine and observed with a microscope. The calcium chloride quantitative values agreed with these qualitative tests while those for the takadiastase and the Walton and Coe methods did not.

But as for most of the tissues the calcium chloride values were lower, in many cases much lower than by the other methods. We are not justified in rejecting the calcium chloride values merely because of this non-agreement. Indeed, on account of the greater selectivity of the calcium chloride method for starch we must consider the possibility that the values by this method are correct and that those by the others are incorrect.

It appears probable that in some of the tissues substances were present which were hydrolyzed by takadiastase (and also by saliva and subsequent acid hydrolysis) but which were not starch. This is certainly true of the tissues containing no starch and may be true of some of the others. Agreement among the methods with such tissues as grape twig, potato tuber, and possibly celery may be explained on the basis that these tissues do not contain appreciable amounts of the non-starch substances hydrolyzable by the enzymes.

Many, probably most, analysts would regard the results with the takadiastase and Walton and Coe methods as undoubtedly too low, the takadiastase values because of incomplete hydrolysis, and the Walton and Coe values because of loss of dextrin in the alcohol precipitate (5, p. 164) and loss of dextrose during the acid hydrolysis (18, 19, 22). Yet, these experiments indicate the possibility that with many tissues the results are too high. The last column in Table IV shows approximately how much too high the takadiastase values may be. The numbers in this column are the differences between the takadiastase and calcium chloride values expressed as a percentage of the takadiastase values. This is on the assumption that the calcium chloride values are correct and that the excess values obtained by takadiastase are due to non-starch substances which are hydrolyzed by takadiastase and calculated as starch. It is seen that commonly 30 to 40 per cent of the starch values by the takadiastase method are due to non-starch substances. And with starch-free tissues or tissues low in starch the proportion of non-starch substances may be 50 to 100 per cent.

DISCUSSION

With a recognition of the importance of pH and of the amount of takadiastase in relation to the amount of starch, we can interpret the results of certain previous investigators who did not find a complete hydrolysis of starch to glucose by takadiastase. Davis and Daish (5) used 0.1 g. of takadiastase with 2.0 grams of starch and this amount of takadiastase is incapable of transforming completely that amount of starch. The proportion used by Thomas (19) was 0.1 g. takadiastase with 0.3 g. starch and this was nearly sufficient for complete hydrolysis. Possibly in this case the pH (which apparently was not controlled) was not favorable for takadiastase action. From the data of Widdowson (22) we find that she used as much as 0.1 g. of takadiastase with tissue samples containing up to about 0.15 g. of starch. The hydrolysis in such a case should have been complete provided that the digestion was carried out at pH 4.5 for at least 44 hours. However, the time of digestion in her experiments was 24 hours, and the only information we have in regard to the acidity was that two drops of 5 per cent acetic acid were added. The pH that was produced by this addition was not stated.

The results of Horton (10) on this point are of especial interest since he produced the most convincing evidence against the reliability of takadiastase as a reagent for the quantitative analysis of starch, showing that the hydrolysis did not proceed to the point at which only maltose and glucose were formed as claimed by Davis and Daish, and even suggesting that in some cases not all of the dextrin had been split. It is easy to see why Horton should have come to this conclusion. He used 0.1 to 0.6 g. of takadiastase with 2.0 to 3.5 g. of starch, amounts of takadiastase quite inadequate for such large amounts of starch. In one experiment, however, Horton (10, p. 249) used 1.0 g. of takadiastase with 1.65 g. of starch, and in this case he approached the proportion at which only dextrose should have been obtained. His data show that he found 1.702 g. of glucose and 0.047 g. of maltose in the products of hydrolysis, which is a close approach to completeness. Also, in his Experiment No. 48 (10, p. 252), when the digestion period was increased to 16 days, 0.5 g. of takadiastase with 2.0620 g. of starch resulted in the formation of 2.1738 g. of dextrose and only 0.0308 g. of maltose. He recognized that increasing the amount of takadiastase generally caused more nearly complete hydrolysis but he was impressed by the several exceptions to this rule. The inconsistencies in the behavior of takadiastase caused him to regard the method as untrustworthy.

It seems likely that the inconsistencies which Horton observed were due to a failure to control the pH value of the digestion mixture. Takadiastase is active in the range pH 3.0 to 5.0. At an unfavorable pH, complete-

ness of hydrolysis would not be obtained, and differences in pH in different tests could cause results that would appear to be inconsistent.

The present experiments upon the completeness of hydrolysis of starch by takadiastase were carried out with potato starch. The results were applied to the plant tissues on the basis that the enzyme would produce complete hydrolysis of the starch of these species also. This point has not been investigated yet and until a quantity of starch grains in approximately pure form have been separated from the tissues, or until tests have been made regarding the absence of dextrin and maltose in the enzyme digests, the calculations will remain subject to correction on this point.

The takadiastase used in these experiments was the commercial preparation of Parke Davis and Co. Potter and Phillips (16) reported that they obtained from the manufacturers a special preparation which was free of sugar and which had about five times the hydrolytic power of the usual product. If such a preparation should become commercially available it would greatly simplify analytical work with starch, and would make unnecessary the purification by dialysis such as was found necessary in the present work.

SUMMARY

Confirmation of previous reports that takadiastase can convert potato starch completely into glucose rather than into a mixture of maltose and glucose was obtained. In order to obtain this result sufficient takadiastase in proportion to the amount of starch must be taken, the pH must be in the range 3.0 to 5.0, and the time of contact must be sufficiently long, approximately 44 hours.

Takadiastase solutions which were dialyzed overnight in collodion bags were free of copper-reducing substances, and retained high amylolytic power. Such solutions were stable at room temperature or at lower temperatures for several weeks.

Long continued boiling of the starch paste previous to adding takadiastase was found to be unnecessary for complete hydrolysis. Indeed, it was not necessary to bring the starch-water mixture even to the boiling point of water; a temperature of 80° C. was sufficient, and only a very slight lowering of hydrolysis occurred at 70°. Polarized light photographs of the starch grains that had been treated with water at different temperatures showed that hydrolysis by takadiastase was related to the condition of the grain at which crosses on the grains were no longer obtained in polarized light.

Starch determinations were made upon the alcohol-extracted, sugar-free, finely powdered tissues from 12 different species of plants, including such plant parts as leaf, stem, tuber, root, fruit, and seed. Most of the tissues were low in starch and some of them were starch-free.

Details are given of a takadiastase method which consisted in digesting the powdered tissue with takadiastase at pH 4.5 for 44 hours, and, after clearing the solution and without hydrolyzing the digest, determining the starch by the increase in copper-reducing power.

Determinations were also made by a method which involved extraction of the starch from the tissues with concentrated calcium chloride solution in which starch is soluble and from which it can be precipitated as starch iodide. The starch in this precipitate was estimated by takadiastase digestion after eliminating the iodine and the CaCl_2 .

With tissues containing no starch appreciable "starch" values were obtained by the takadiastase method, but with the CaCl_2 method zero or very low values were obtained.

With most of the tissues the CaCl_2 method gave much lower values. This is believed to indicate that the takadiastase values were too high and that non-starch substances in the tissues were hydrolyzed by the enzyme and were estimated as starch. These non-starch substances commonly represented 30 to 40 per cent of the values computed as starch by the takadiastase method, and in some cases they represented 50 to 100 per cent.

A few of the tissues showed the same or very similar values by both methods. This is interpreted as indicating that in these tissues non-starch substances hydrolyzable by takadiastase were not present.

Confirmation was not obtained of the view commonly held that glucose or other hydrolytic products of starch are destroyed by heating with acid under the conditions for acid hydrolysis of starch. Destruction of sugar as indicated by lowered copper-reducing power was obtained only when the amount of acid was more than twice that usually recommended for acid hydrolysis of starch.

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EFFECT OF VARIOUS FACTORS ON THE SOLUBLE MANGANESE IN SOILS

M. M. McCool

There are certain soil types on the property of the Boyce Thompson Institute for Plant Research in which tomato, tobacco, soybean, and buckwheat do not thrive under greenhouse conditions. These soils are less productive after they have been subjected to steam under pressure in preparing them for various experimental purposes. While studying methods to improve these soils it was ascertained that the manganese in the water extracts of steam heated soils and that of plants grown in them was strikingly high. As a result, investigations on the water soluble manganese present at different times of the year, under different water contents, temperatures, lime, and fertilizer treatments have been conducted. The amount of manganese brought into solution by treating a large number of soils with acetic acid has been determined.

REVIEW OF LITERATURE

Comprehensive reviews of literature on the various manganese relationships of soils and plants have been presented recently by Carlyle (1), Emmert (4), Jacobson and Swanback (6), Mann (9), and Willis (14). Only the available literature dealing with closely related subjects included in these investigations is referred to in this report.

Funchess (5) found an accumulation of manganese in November, at or near the surface of a Georgia soil. He also reported that the nitrification of cotton seed meal, dried blood, and ammonium sulphate was accompanied by an increase in water soluble manganese. According to Robinson (11) the solubility of manganese in soil is affected greatly by oxidizing and reducing conditions. He found the amount of manganese in solution to be increased greatly by submerging soil in water several days in tightly stoppered bottles. Kelley and McGeorge (8) reported that the water soluble manganese was increased in several soils by air drying and by drying them at 100° C. and 250 ° C., respectively.

It was brought out that the soil reaction (2, 3, 4, 5, 11, 12, 13) is an important factor in regulating the water soluble manganese of a given soil. The amount was reduced by raising the pH values. Judging from the results reported by Mann (9) however, there may not be a direct correlation between the pH values of soils and their soluble manganese content since it was reduced to extremely small amounts by bringing the pH value of a sample of Dunbar fine sandy loam to 5.8 with lime; but with Dunkirk gravelly sandy loam this did not result until pH 7.0 was reached.

It has been shown (2, 3, 10) that some soils contain replaceable manganese. Thus, increases in the amount of this base in the soil solution may be due in part to its displacement by soluble salts.

MATERIALS AND METHODS

The soils employed, except the Merrimac sandy loam, Podunk silt loam, Dutchess silt loam, Dover fine sandy loam, Dover silt loam, and Gloucester loam, were obtained through the courtesy of Dr. C. F. Marbut. Three plots of ground about ten feet square were set aside in the spring for studying the water soluble manganese. The surface layers of the soil were thoroughly mixed before the tests were begun. The samples were taken at the depths and on the dates given in Table I. About 600-gram samples were collected and the water content determined. After air drying, one part of soil and five parts of water were placed in the cup of a Bouyoucos electrical dispersing machine, stirred ten minutes, transferred to a filter paper in a Büchner funnel, and filtered by suction. A suitable aliquot was taken, reduced to a small volume, and the manganese content determined. Unless otherwise stated the same methods of procedure were followed throughout in obtaining soil extracts. In determining the effect of temperature and water content of soils on the water soluble manganese, duplicate 200-gram portions were placed in Erlenmeyer flasks of 500-cc. capacity, the flasks were loosely plugged with cotton and maintained at different but constant temperatures. The ammonium sulphate-treated soils were maintained at room temperatures.

When large volumes of soil were required in the studies on the effect of heat on the soluble manganese content of soils, a large doubled-walled sterilizer was utilized. The temperature was maintained at 240° F. and the pressure at eight pounds for three hours. The soils were placed in potting frames and run into the chamber on a track. A laboratory autoclave was employed in treating small amounts of soil.

The sodium chlorate-potassium periodate method (4) was used in determining the manganese content of soil extracts and plants.

The quinhydrone electrode was employed in making pH determinations. The ratio of water to air-dry soil was 1 to 3 for field samples and 1 to 5 in the others. The readings were taken ten minutes after the water was added.

The displaced soil solutions were obtained by packing four pounds of moist soil in iron tubes 4 inches in diameter and 18 inches in length. Water was added above the soil and pressure applied. The first 50 cc. of the displaced solution were discarded.

Acetic acid soluble manganese was determined on five grams of air-dry soil. The soil was placed in 1000 cc. of one-fifth normal acetic acid. The mass was shaken by hand, one minute each hour for five hours, filtered,

TABLE I
WATER SOLUBLE MANGANESE IN FIELD SOILS AT DIFFERENT TIMES OF THE YEAR

Soil type	Depth of sampling in inches	Water per cent					pH		P.p.m. manganese dry soil basis				
		May 12	June 16	July 18	August 1	Sept. 18	June 16	Sept. 18	May 12	June 16	July 18	August 1	Sept. 18
Merrimac sandy loam	0-1/4	5.4	6.8	5.5	7.2	16.4	4.9	4.9	1.0	0.9	2.4	4.3	2.2
	1/4-6	8.2	10.2	10.4	9.4	14.5	5.0	4.8	Trace	Trace	1.3	2.6	1.8
	6-12	14.0	13.4	12.6	10.8	15.4	5.2	4.9	Trace	Trace	0.4	1.2	0.9
	12-18	18.0	17.5	18.2	18.4	17.0	5.3	5.3	Trace	Trace	Trace	0.4	0.5
Merrimac sandy loam sod mixed with surface 6 inches	0-1/4	6.4	7.8	6.2	8.4	22.0			6.4	5.6	7.8	12.7	4.2
	1/4-6	9.4	15.8	10.2	9.6	22.1			3.5	2.8	6.4	7.4	6.4
	6-12	14.5	14.0	15.0	9.4	16.5			1.4	1.2	0.8	4.6	2.8
	12-18	18.0	18.4	17.4	18.0	18.6			Trace	0.5	0.4	0.5	0.4
Podunk silt loam	0-1/4	6.3	8.4	4.7	6.3	20.0	5.1	5.0	3.7	1.4	3.0	6.0	2.3
	1/4-6	10.6	12.5	9.5	7.7	20.0	5.0	5.0	1.4	0.8	0.5	5.5	3.7
	6-12	13.4	16.4	14.9	0.5	21.2	5.1	5.1	1.1	0.6	0.3	3.5	2.6
	12-18	17.8	18.2	18.0	18.8	18.4	5.3	5.2	0.6	Trace	0.4	0.5	0.6

and filtrate evaporated to a small volume, and the manganese content determined.

RESULTS

Water soluble manganese in field soils at different periods. The amount of manganese in the extracts of samples taken from field soils at different depths from the surface at different times of the year was determined. The soils were kept free of vegetation during the progress of the experiment. The results are given in Table I. Variations were found in the manganese content of the water extracts of samples of soil taken at different depths from the surface and at different times of the year. It was lowest in spring and after heavy precipitation and in the autumn. The maximum amounts were found near the surface in August and in the soil which contained decaying sod. The variations in the water soluble manganese may be attributed to differences in the temperature of the soil, to carbon dioxide production, to upward and downward movements of water, and to the displacement of it by the bases of salts which are brought into solution by various factors. It is conceivable that the amount of manganese in the soil solution of some acid soils would reach relatively large proportions during a prolonged period of high temperature and sufficient rainfall to maintain them in a moist condition with a minimum amount of leaching.

Effect of water content of soils on their water soluble manganese contents. Inasmuch as soils may be stored and utilized for plants growth with different amounts of water in them, it seems advisable to determine if there is any relationship between the water content at which soils are held and their water soluble manganese content. Samples of Podunk silt loam and Merrimac sandy loam were held at temperatures ranging from 75° to 80° F. for the intervals of time and at the water contents given in Table II. At the end of each period the amount of manganese extracted with water was determined. Although the experiment was of short duration the high water content was more effective in the release of manganese than

TABLE II
EFFECT OF WATER CONTENT ON SOLUBLE MANGANESE IN SOILS

Days held	Podunk silt loam			Merrimac sandy loam	
	10% water	35% water	60% water	17% water	60% water
	P.p.m. Mn in dry soil	P.p.m. Mn in dry soil	P.p.m. Mn in dry soil	P.p.m. Mn in dry soil	P.p.m. Mn in dry soil
1	2.7	3.0	2.9	2.3	2.5
2	2.6	2.7	2.6	2.6	6.5
7	3.0	3.4	5.5	4.3	8.3

were the lower ones. It appears that if some soils having a high water content were exposed to high temperature, such as may take place in greenhouses and storage bins, manganese would accumulate in the soil solution in rather large amounts. This is exemplified by the next set of experiments.

Effect of storing soils at different temperatures on their water soluble manganese contents. The amount of manganese in extracts from moist soil held different periods of time at 35° F., 72° F., and 100° F. was determined. According to the data in Table III changes in the water soluble manganese in the soils exposed to 35° F. were slight. At this temperature the maximum amount was obtained from the Dover silt loam and the Dutchess silt loam at the end of the three-day period. The samples exposed to 72° F. carried the most water soluble manganese at the close of the 30-day period. The quantity increased in each of the soils held at 100° F. as the experiment progressed.

TABLE III

EFFECT OF TEMPERATURE ON THE WATER SOLUBLE MANGANESE IN SOILS; RESULTS EXPRESSED IN PARTS PER MILLION OF DRY SOIL

Soil type	Temperature								
	35° F.			72° F.			100° F.		
	3 days	15 days	30 days	3 days	15 days	30 days	3 days	15 days	30 days
Dover silt loam	2.9	0.5	0.5	3.4	1.8	4.4	3.3	3.8	12.7
Dutchess silt loam	0.7	0.3	0.3	0.6	0.8	1.1	0.8	—	3.4
Podunk silt loam	0.4	0.5	0.7	0.4	0.7	1.8	0.5	2.5	4.6

The manganese content of extracts of turf and adhering soil, taken to a depth of about four inches, in Merrimac sandy loam, stored about six months in a large mass at room temperature with a moisture content of 20 per cent, contained 30.8 parts of manganese per million of dry soil. Podunk silt loam, water content 16 per cent, after having been stored 120 days contained 9.7 parts of manganese per million of dry soil.

The above results suggest the possibility of a sufficient accumulation of this element in the soil solution of acid soils, when stored in the moist condition and when used in the greenhouse under high temperatures, to be harmful to some kinds of plants.

Effect of steam heating on the soluble manganese content of soils. The effect of steam heating moist soils on the amount of manganese in water extracts of six soils, 1, 15, 30, and 60 days after treatment, was studied. The soils were heated in the laboratory autoclave and stored at room temperature in Erlenmeyer flasks loosely plugged with cotton. According

to the data in Table IV rather large amounts of manganese were liberated by this treatment. The maximum amount was present one day subsequent to heating. The quantity found in each of the extracts 14 days later was much less. From the close of this to the next or 30-day period additional

TABLE IV
EFFECT OF STEAM HEATING ON THE WATER SOLUBLE MANGANESE CONTENT OF SOILS

Soil type	P.p.m. of manganese dry soil basis, time in days after heating			
	1	15	30	60
Dutchess silt loam	51.9	23.1	20.4	12.3
Dover silt loam	104.1	45.5	40.0	30.7
Dover fine sandy loam	25.2	13.6	11.9	6.6
Gloucester loam	56.8	38.0	27.7	—
Merrimac sandy loam	31.7	15.7	15.0	16.9
Podunk silt loam	37.0	21.7	27.3	5.7

but smaller reductions took place, except in the Podunk silt loam and Merrimac sandy loam extracts. The manganese content of the Podunk silt loam extract was greater and that of the Merrimac sandy loam extract was practically the same as it was at the end of the previous period. At the close of the experiment less manganese was present in all extracts with the exception of the Merrimac sandy loam. It is likely that the water content, temperature, and aeration, under which heated soils are stored, influence the changes that take place in their manganese content.

Samples taken 12 to 24 and 24 to 36 inches from the surface of Dover silt loam and Dutchess silt loam, and 12 to 18 inches from the surface of Podunk silt loam and Merrimac sandy loam, were heated in the usual manner and the soluble manganese determined. With the exception of the sample taken from the 24 to 36-inch section of the Dover silt loam, the quantity of manganese brought into solution was minute. As is shown in Table VIII these contained relatively large amounts of acetic acid soluble manganese. It would appear from these results and the field studies on Merrimac sandy loam that organic matter is important in the liberation of manganese to the soil solution.

The manganese content of displaced solutions from the above heated soils was determined. It should be noted that about 72 hours elapsed from the time of heating until the solutions were obtained. The data in Table V show that the amount present in the solution displaced from Dutchess silt loam, Dover silt loam, and Merrimac sandy loam was very large. It should be noticed also that steam heating the less acid soils or Dutchess silt loam and Dover silt loam lowered the pH values of their displaced solutions to a greater extent than it did those of the more acid ones or Podunk silt loam, Merrimac sandy loam, and Gloucester loam.

TABLE V
MANGANESE CONTENT AND pH OF DISPLACED SOLUTIONS FROM UNHEATED AND
HEATED SOILS

Soil type	pH		Manganese p.p.m. solution	
	Not heated	Heated	Not heated	Heated
Dutchess silt loam	6.3	5.7	13.8	269.2
Dover silt loam	6.4	5.7	27.1	384.6
Podunk silt loam	5.1	4.9	2.2	22.9
Merrimac sandy loam	4.4	4.1	7.4	186.0
Gloucester loam	5.6	5.2	6.7	117.6

These results are in accord with those which Kelley and McGeorge (8) reported from their investigations on the effect of drying soils at different temperatures.

Samples were placed in sausage skins and dialyzed in running water overnight and the manganese present in the residue was determined. None of the manganese remained after this treatment.

Effect of calcium hydroxide on the manganese content of extracts of heated soils. Calcium hydroxide was added to six soils before heating and the effect on the amount of manganese brought into solution was determined. The amount of hydrate applied, the pH values of the soils, and the soluble manganese are given in Table VI. The pH values were determined after the soils had been dispersed ten minutes in the dispersing machine and just before filtering. It is to be observed that each addition of the hydrate to these soils before heating decreased the amount of manganese in the extracts. The addition of one part to 1000 parts of Podunk silt loam reduced the manganese content more than one-half, one part to 500 to less than one-sixth of that in the unlimed soil extract. There was no manganese in the alkaline extract of the sample treated with one part of hydrate to 250 parts of soil. Similar but less striking results were obtained with the sample of Merrimac sandy loam. Change in the pH value of this soil from 4.1 to 6.9 caused a decrease of manganese from 71.4 to 8.6 parts per million of the soil. The addition of one part of calcium hydroxide to 250 parts of Dover fine sandy loam raised the pH from 5.7 to 8.0 and reduced the manganese from 31.1 parts per million of soil to only a trace. Although the hydroxide was effective in lowering the soluble manganese in the extracts from Dover silt loam, Dutchess silt loam, and Gloucester loam, the largest application did not prevent some of the manganese from going into solution. Thus there remained some manganese in the extracts from three soils made neutral or alkaline in reaction. It should be noted in this connection that Johnson (7) reported the injury to plants grown in heated soils to be most marked in acid soils and the neutralization of the soil with calcium carbonate before treatment removed its toxic property. Although the ef-

TABLE VI
EFFECT OF CALCIUM HYDROXIDE ON THE MANGANESE CONTENT AND PH OF EXTRACTS
TRACTS FROM HEATED SOILS

Soil type	Calcium hydroxide soil ratio	pH	Manganese dry soil
Podunk silt loam	0	4.7	38.0
	1-1000	—	16.0
	1-500	5.8	6.1
	1-250	7.8	0
Merrimac sandy loam	0	4.1	71.4
	1-1000	4.5	44.5
	1-500	5.0	30.0
	1-250	6.9	8.6
Dover fine sandy loam	0	5.7	31.1
	1-1000	6.6	17.0
	1-500	6.8	6.7
	1-250	8.0	Trace
Dover silt loam	0	5.6	130.7
	1-1000	6.1	77.8
	1-500	6.7	49.1
	1-250	7.5	17.7
Dutchess silt loam	0	5.6	111.1
	1-1000	6.4	63.9
	1-500	6.5	40.0
	1-250	7.4	13.1
Gloucester loam	0	5.4	95.7
	1-1000	6.0	57.3
	1-500	6.5	36.2
	1-250	7.0	10.0

fect of heating the soils on the water soluble manganese in them was not determined, it is probable that it was increased thereby and, if so, the limestone retarded the accumulation of the manganese in the soil solution. This might have accounted, in part at least, for the reduction in toxicity of the heated soils.

Effect of ammonium sulphate, mixed fertilizer, and superphosphate on the amount of water soluble manganese in soils. Funchess (5) incubated several ammonium sulphate-treated soils 46 days and attributed the increase of manganese in the soil extracts to the nitrification of this salt and its direct effect. In order to ascertain if base exchange took place, Merrimac sandy loam and Podunk silt loam were treated with different amounts of ammonium sulphate and the manganese content of soil extracts determined 1, 10, and 39 days after its addition to the soils. According to the data in Table VII manganese was rendered soluble soon after the addition of the ammonium sulphate. Upon incubation 10 days there was no increase in the amount of manganese in the soil extract over that present one day

after its application. At the end of 39 days, however, it was much higher. It is apparent that base exchange took place and it is also probable that biological activities caused an increase in soluble manganese.

TABLE VII
EFFECT OF AMMONIUM SULPHATE ON THE WATER SOLUBLE MANGANESE IN SOILS

Ammonium sulphate added	Merrimac sandy loam			Podunk silt loam		
	Manganese p.p.m. of dry soil			Manganese p.p.m. of dry soil		
	1 day	10 days	39 days	1 day	10 days	39 days
Check, no treatment	2.1	2.2	7.0	2.6	2.5	5.0
165 p.p.m. of soil	3.0	3.3	12.5	3.6	3.8	13.9
330 p.p.m. of soil	4.3	4.2	13.6	4.0	4.0	13.5
660 p.p.m. of soil	5.6	5.7	13.0	4.8	4.8	18.5

A 4-8-7 commercial fertilizer and superphosphate, respectively, were added to the above soils before heating. One part of the former was added to 100 parts of the soil and one part of the phosphate was mixed with 70, 140, and 280 parts of the soils. The manganese content of the water extracts was increased by the addition of these fertilizers.

Acetic acid soluble manganese in soils. Although it appears that the amount of manganese removed from soils by some plants is influenced by other elements in the soil solution it may be that there is some relationship between the amount of acetic acid soluble manganese in soils and that removed by plants. Samples taken at different depths from the surface of 18 widely distributed soil types were extracted with fifth normal acetic acid and the quantity of manganese in the solutions determined. The results of these studies are summarized in Table VIII.

Small amounts of manganese were found in acetic acid extracts of Leonardtown silt loam, Portsmouth loam, Orangeburg sandy loam (except the surface layer which was mainly organic matter), the first three sections of Ontonagon clay loam, the second, third, and fourth of Gloucester fine sandy loam, fourth, fifth, sixth, and seventh of the Norfolk sandy loam, and the second of the Miami and Dutchess stony loam.

Manganese content of plants grown in steamed and unsteamed soils. Several kinds of plants grown in heated soils developed leaf characteristics similar to, if not identical with, those which resulted when they were produced in soils to which liberal amounts of manganese sulphate were added. In preliminary studies, moreover, it was found that the leaves of the plants grown in the former contained much more manganese than did those taken from plants grown in unsteamed soils. In addition, the application of commercial fertilizer prevented the injury to several plants.

The fields of the first, second, and third crops of soybean (*Glycine max*

TABLE VIII
AMOUNT OF MANGANESE SOLUBLE IN ONE-FIFTH NORMAL ACETIC ACID IN SOILS

Soil type and location	Depth from surface in inches	pH	Manganese p.p.m. of dry soil	Soil type and location	Depth from surface in inches	pH	Manganese p.p.m. of dry soil
Leonardtown silt loam, Colvert Co., Md.	0-1	3.7	Trace	Portsmouth loam, Gates Co., Ga.	6-12	3.7	Trace
	1-8	4.3	11.1		12-20	4.6	Trace
	8-22	4.4	Trace		20-44	4.3	Trace
	22-38	4.9	Trace	Orangeburg sandy loam, Jefferson Co., Ga.	0-2	5.6	104.7
	38-50	4.9	Trace		2-10	5.4	Trace
Ontonagon clay loam, Luce Co., Mich.	50+	5.0	Trace		10-20	5.0	Trace
	2-6	5.6	Trace		20-60	5.0	Trace
	6-12	5.6	13.3		60-100	4.5	Trace
	12-30	6.3	61.3		100+	4.5	0
Sassafras silt loam, Cecil Co., Md.	0-4	5.7	56.0	Chester loam, Cecil Co., Md.	0-1½	4.8	63.6
	4-12	5.1	43.2		1½-8	4.5	142.8
	12-20	5.3	31.6		8-32	4.6	59.2
	20-38	5.2	Trace		32-44	4.7	Trace
	38+	5.7	Trace		44-60	5.0	Trace
Fox loam, Eaton Co., Mich.	0-3	6.2	103.0	Gloucester fine sandy loam, Hampden and Hampshire Cos., Mass.	0-5	4.2	90.0
	3-14	6.2	51.4		6-15	4.9	10.4
	14-36	6.3	47.1		15-20	5.3	Trace
	36-48	7.6	99.0		20-36	5.7	Trace

TABLE III (Continued)

Canfield silt loam, Delaware Co., N.Y.	0-7	4-5	36.2	Dutchess stony loam, Rennselaer Co., N. Y.	0-6	5.4	92.6 Trace
	7-12	4.7	21.4		6-15	4.7	
Miami loam, Eaton Co., Mich.	12-22	4.9	97.8	Schoy loam, McKenzie Co., N.D.	15-24	4.9	28.5
	22-40	4.7	125.6		24-36	5.4	16.6
	40-75	5.4	40.8		0-2	6.1	86.2
	0-2	5.5	25.3		2-5	5.9	68.2
Onaway loam, Montmorency Co., Mich.	2-8	5.0	11.1	Merrimac sandy loam, Westchester Co., N. Y.	5-14	6.4	47.9
	16-36	6.2	47.4		14-22	7.0	50.0
	36-50	8.0	125.6		22+	7.9	132.0
	2-6	7.5	40.8		0-6	4.9	33.3
Norfolk sandy loam, Jefferson Co., Ga.	6-15	7.2	Trace	Podunk silt loam, Westchester Co., N. Y.	6-12	4.8	12.0
	15-30	6.3	35.2		12-18	5.3	4.8
	30-48	7.9	21.2		0-6	5.1	21.8
	0-2	4.9	421.1	Dover silt loam, Dutchess Co., N. Y.	6-12	5.1	15.0
	2-6	5.1	144.9		12-18	5.3	23.8
	6-12	5.1	36.2		0-8	5.6	128.0
	12-18	5.5	Trace	Dutchess silt loam, Dutchess Co., N. Y.	12-24	6.1	31.0
	18-30	5.8	o		24-36	5.7	44.3
	30-60	5.1	Trace		0-8	6.1	106.0
	60-72	4.9	Trace		12-24	5.9	37.0
					24-36	6.4	33.0

Merr.) and those of buckwheat (*Fagopyrum esculentum* Moench.) which followed the third crop of soybeans are given in Table IX and illustrated by Figures 1 and 2. The plants were grown in two-gallon glazed jars. The

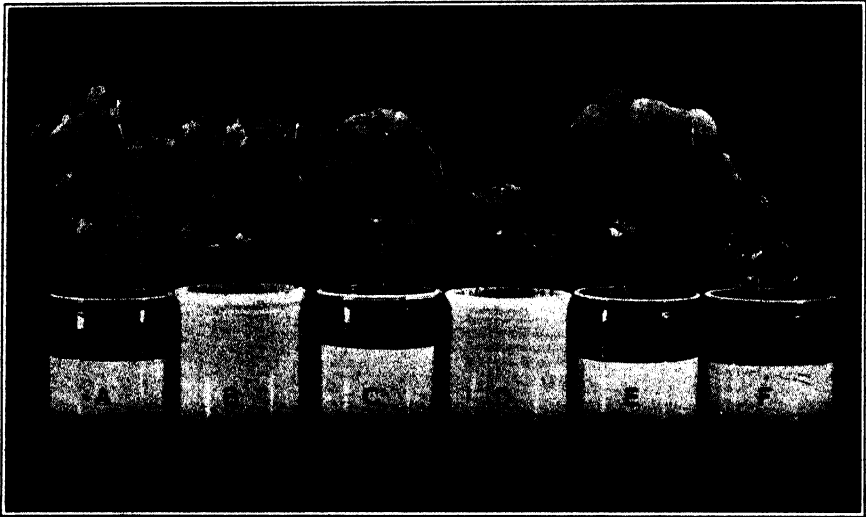


FIGURE 1. Soybeans growing in unsteamed and steamed soils. A and B, Dover silt loam; C and D, Gloucester loam; E and F, Merrimac sandy loam. Soils in A, C, and E were not steamed and those in B, D, and F were steamed.

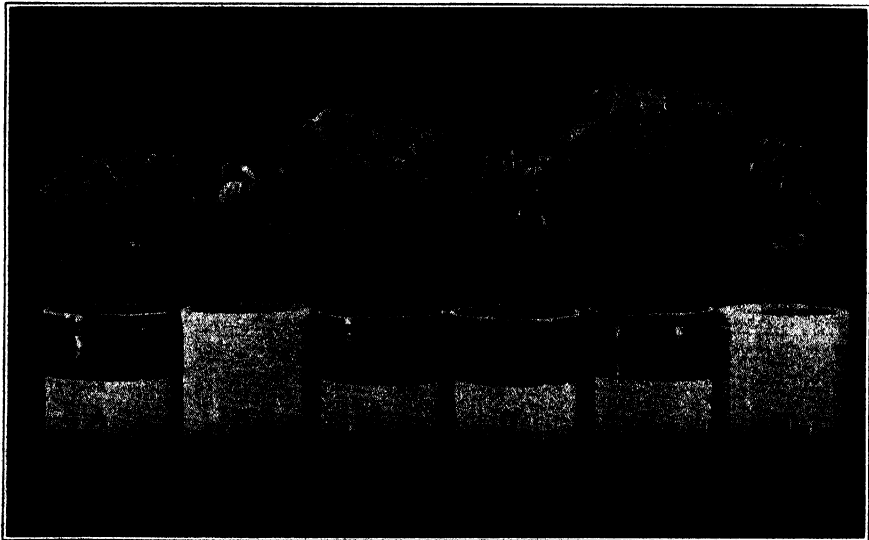


FIGURE 2. Soybeans growing in unsteamed and steamed soils. A and B, Dover fine sandy loam; C and D, Dutchess silt loam; E and F, Podunk silt loam. Soils A, C, and E were not steamed and those in B, D, and F were steamed.

TABLE IX
GROWTH OF SOYBEAN AND BUCKWHEAT IN UNSTEAMED AND STEAMED SOILS; HARVESTED AT FIRST BLOOM STAGE

Soil type	Soybean						
	First crop		Second crop		Third crop		Buckwheat following 3rd crop of soybean
	Fresh wt., g.	Leaf injury	Fresh wt., g.	Leaf injury	Fresh wt., g.	Leaf injury	
Dover silt loam	83.4		64.5		53.5		Fresh wt., g. 41.6
Dover silt loam steamed	71.2	Great	39.0	Great	32.6	Great	27.0 Great
Dover fine sandy loam	114.2		64.0		44.0		30.6
Dover fine sandy loam steamed	47.2	Great	50.0	Slight	42.5	Medium	29.2 Slight
Dutchess silt loam	86.6		76.0		55.0		57.0
Dutchess silt loam steamed	59.0	Great	39.3	Great	40.0	Medium	19.2 Great
Podunk silt loam	130.6		64.7		54.2		
Podunk silt loam steamed	52.2	Great	55.0	Medium	42.5	Medium	22.8 Great
Gloucester loam	100.0		59.0		44.6		
Gloucester loam steamed	33.2	Great	40.0	Great	24.2	Great	8.5 Great
Merrimac sandy loam	82.0		55.7		43.0		32.1
Merrimac sandy loam steamed	29.2	Great	55.5	Medium	44.5	Medium	15.4 Great

soils in one set of duplicate cultures were steamed. After each crop of soybeans was harvested the roots were carefully removed and seed planted. The degree of leaf injury to the plants resulting from steaming the soils is also given in this table. The leaf characteristics of injured soybean plants consisted of darkening of the veins, the appearance of brown spots, and curling. The injury to the leaves of buckwheat plants was evidenced by pale green, yellowish, and nearly white color and also leaf curl. These, depending upon the degree of injury, are grouped as great, medium, and slight. The root development of the plants grown in the steamed soils was much less, with a greatly lessened number of fibrous roots in comparison with those produced in the unsteamed soils.

An examination of the yields shows the production of soybeans to have decreased with each succeeding crop in the untreated soils. The development in the steamed soils was less than it was in the untreated ones with the exceptions of the second and third crops in the Merrimac sandy loam, the third ones in the Dover fine sandy loam, and the Merrimac sandy loam. Relatively low yields of buckwheat were obtained in the steamed soils, with the exception of the Dover fine sandy loam.

The manganese content of the leaves of soybeans varied widely with the soil in which they were grown. It increased in amount with each succeeding crop with the exception of the second crop in Dover fine sandy loam. The leaves of the plants produced in the steamed soils contained large amounts of the element and without exception the second crop carried more than the first and less than the third.

It was found by these and other tests that there was more manganese in the leaves of buckwheat than there was in those of soybeans, that is, when grown under similar conditions. The leaves of buckwheat plants obtained from the steamed series of soil cultures were remarkably high in manganese. It ranged in amount from 0.575 per cent in those grown in the Dover fine sandy loam to 2.251 per cent in those taken from the Gloucester loam. It is probable that the increase in the amount of manganese in the succeeding crops was due in part to a diminished supply of available nutrients and decreased plant vigor.

The water soluble manganese content of the soils immediately following the treatment with steam and after the removal of the different crops of soybean as given in Table X should be noted. There was a striking decrease in the amount of this element in the extracts of steamed soils after the removal of the first crop. The quantity extracted from the steamed samples of Dover silt loam, Dutchess silt loam, and Gloucester loam taken after the harvest of the second crop, was much greater than that obtained from the previous one. The water extractable manganese in the steamed samples of Dover silt loam, Dover fine sandy loam, and Dutchess silt loam after the removal of the third harvest of soybeans was about the same

TABLE X
WATER SOLUBLE MANGANESE CONTENT OF STEAMED AND UNSTEAMED SOILS AND TOTAL CONTENT OF LEAVES
OF SOYBEAN AND BUCKWHEAT PLANTS HARVESTED AT FIRST BLOOM STAGE

Soil type	P.p.m. manganese in soil, dry basis				% manganese in dry leaves			
	Before planting	After 1st crop of soybeans	After 2nd crop of soybeans	After 3rd crop of soybeans	1st crop of soybeans	2nd crop of soybeans	3rd crop of soybeans	Buckwheat after 3rd crop of soybeans
Dover silt loam	2.90	13.2	13.15	—	0.095	0.130	0.156	0.222
Dover silt loam steamed	104.00	70.0	25.31	65.0	0.233	0.460	0.606	1.377
Dover fine sandy loam	0.36	1.5	1.11	—	0.017	0.016	0.024	0.045
Dover fine sandy loam steamed	25.00	12.7	10.47	13.3	0.130	0.154	0.261	0.575
Dutchess silt loam	0.70	6.4	13.55	—	0.039	0.061	0.082	0.184
Dutchess silt loam steamed	51.96	64.5	11.90	58.1	0.226	0.454	0.519	1.499
Podunk silt loam	0.45	5.7	6.35	—	0.040	0.052	0.073	—
Podunk silt loam steamed	37.00	23.0	26.00	33.2	0.198	0.353	0.446	1.534
Gloucester loam	0.40	3.8	4.37	—	0.039	0.054	—	—
Gloucester loam steamed	56.80	50.7	24.41	71.1	0.268	0.657	0.820	2.251
Merrimac sandy loam	0.85	6.4	5.89	—	0.027	0.053	0.106	0.200
Merrimac sandy loam steamed	31.70	18.4	14.70	6.1	0.184	0.264	0.386	1.500

as that obtained from the previous set, but the amounts in those taken from the Podunk silt loam and Gloucester loam were markedly greater. The quantity was less in the Merrimac sandy loam. The unsteamed soils contained the smallest amount of water soluble manganese before plants were grown in them.

These results are interesting in that they reveal, with one exception, the continuance of a relatively high water soluble manganese content of the steamed soils. This fact coupled with the high manganese content of the leaves of soybeans and buckwheat grown in them, together with the development of leaf characteristics similar to those which occurred when these were grown in soils to which large applications of manganese sulphate were added are suggestive. They indicate, if they do not prove, that the release of manganese to the soil solution at least was a contributory factor in the unsatisfactory development of plants in the steamed soils. Additional studies on manganese, soil, and plant relationships are in progress.

SUMMARY

1. The manganese content of water extracts of samples of soil taken at different depths from the surface and at different times of the year varied widely. It was lowest in spring after heavy precipitation and in the autumn. The largest amounts were present near the surface in August and in soil which contained decaying sod.

2. More manganese was present in extracts of soils held at high water contents than was contained in those from soils maintained at lower water contents.

3. Changes in the amount of manganese in extracts from soils held at different periods of time, or 3, 15, and 30 days at 35° F., were slight. The quantity of water soluble manganese in the soils held at 72° F. and 100° F. increased as the experiment progressed and it was greatest in those maintained at the latter temperature.

4. Steam heating six soils three hours at 240° F. greatly increased the manganese in their soil extracts. Upon standing at room temperature 15 days, the quantity in solution decreased greatly. The subsequent changes, or 30 and 60 days after treatment, were not so great. The soluble manganese content of samples taken from the subsoils of Dover silt loam, Dutchess silt loam, Podunk silt loam, and Merrimac sandy loam, with the exception of the one removed 24 to 36 inches from the surface of Dover silt loam, was increased only to a slight extent by steaming. It would appear that organic matter played an important rôle in the release of manganese to the extracts upon steaming these soils. The manganese content of solutions displaced from six steamed soils was very high. It ranged in amount from 384 parts per million in that from the Dover silt loam to 22.9 parts per million in that from the Podunk silt loam.

5. The addition of different amounts of calcium hydroxide to six soils before steaming resulted in great decreases in the water soluble manganese in comparison with that present in samples of these soils which did not receive the hydroxide. Although the extracts of three of the soils were alkaline in reaction they carried some manganese.

6. The addition of ammonium sulphate, superphosphate, and mixed fertilizer to Merrimac sandy loam and Podunk silt loam increased the amount of manganese in their soil extracts.

7. Samples taken at different depths from the surface of 18 soil types were digested with one-fifth normal acetic acid and the amount of manganese brought into solution determined. Great differences in the quantity of this element released to the acid were obtained. Several samples gave only traces whereas others released one hundred or more parts per million of soil.

8. Plants grown in steamed soils contained much larger amounts of manganese and developed leaf characteristics similar to, if not identical with, those produced in soils to which excessive amounts of manganese were applied. The harmful action of steamed soils towards several kinds of plants was prevented by commercial fertilizer.

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A DEVICE FOR UNIFORM LIGHTING IN PRECIPITIN TESTS

HELEN PURDY BEALE

In determining end points for the precipitin reaction by the serologic technique, the precipitate in the final tubes of a series may be so slight as to escape detection at times, if varying conditions of lighting are employed. The accompanying diagram, Figure 1, represents a box, designed to provide uniform lighting conditions for reading precipitin tests.

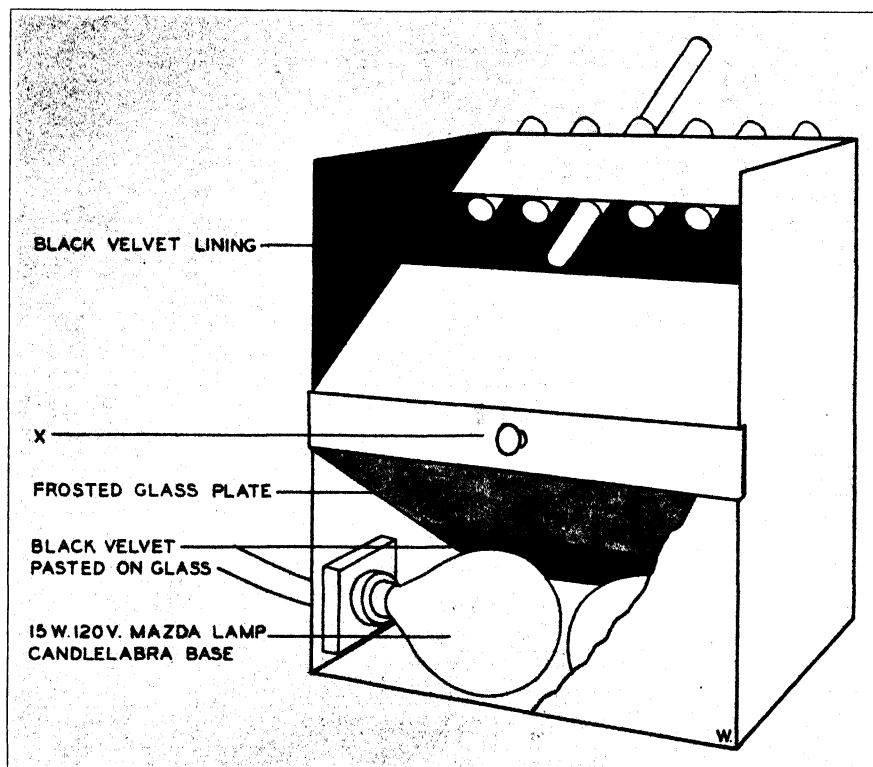


FIGURE 1. Apparatus for convenience and accuracy in observing precipitates formed in precipitin reaction.

The box is constructed of aluminum, with a base 3.5 in. \times 5.0 in. and a height, at the back and sides, of 5.5 in. A sloping front top-piece is detachable at screw x to allow access to the bulbs. A frosted glass plate, at an angle indicated in the diagram, disperses the light from the two Mazda lamps (15 watt, 120 volt with candleabra base) in the chamber below to

the upper portion of the box. The upper compartment is lined with black velvet and a strip of the velvet is pasted along the lower end of the upper surface of the glass plate in order to eliminate reflection and provide a dark background.

The holders for the precipitin tubes are soldered in position and the lower ends are slit to allow a certain flexibility upon insertion of the tubes. Due to lack of uniformity in diameter, the tubes do not always fit the holders tightly. This may be remedied conveniently by slipping a narrow cross section of rubber tubing of an appropriate diameter over the precipitin tube before insertion in the holder to support it in place at the desired level.

Due to the unequal heating of the tubes on the lower side by the heat rising from the bulbs, a slow circulation of the tube contents begins, which is most helpful in reading the reaction in tubes in which the flocculi have settled out in the bottom. The reaction is recorded shortly after the circulation sets in, so that the possible effect of increasing temperature on precipitation of the tube contents is negligible as a source of error.

The apparatus described here accommodates six precipitin tubes, but the size of the box can be readily altered to include a larger number of holders.

Such a lighting box not only enables the individual investigator to read his precipitin tests with greater facility and accuracy, but it likewise offers a standardized method to independent investigators for reading precipitin tests under uniform conditions of lighting.

PLANT INJURY CAUSED BY VAPORS OF MERCURY AND COMPOUNDS OF MERCURY

P. W. ZIMMERMAN AND WILLIAM CROCKER

In the spring of 1932 the authors were asked to diagnose a peculiar type of injury on Briarcliff roses grown by a florist in Attleboro, Massachusetts. The information at hand concerning management of the rose houses was that the fertilizing and fumigating had been done as in previous years. The only variation was an application of bichloride of mercury to the soil of some of the several beds where earthworms had been particularly bad. The rose injury, however, extended over the entire rose house. The petals from partially opened buds were brown, the corollas of younger buds had turned brown and abscised without opening, stamens were killed, and peduncles were injured and had turned dark brown or nearly black in places. An abnormally large number of old leaves had fallen and a considerable number of those remaining on the plants were brown in places. The injuries in general did not coincide with responses known to be caused by fertilizers, insecticides, fungicides, or illuminating gas, all of which had been considered. Evidently the only way to solve the problem was to conduct experiments under controlled conditions with the different materials under suspicion. The investigation was started in the rose house at the Boyce Thompson Institute on March 22, 1932 by treating the soil of one bed of two-year-old rose plants with bichloride of mercury. Injuries similar to those described above occurred over the entire house though the soil of only one of the four beds had been treated. The evidence pointed to mercury as the probable toxic ingredient and led to experiments where potted rose plants were confined in glass cases, and treated as follows: No. 1 enclosed with soil moistened with a solution of bichloride, No. 2 enclosed with a pan of metallic mercury, and No. 3 enclosed without treatment as controls. The leaves and flowers of all plants in cases Nos. 1 and 2 were severely injured while the controls in No. 3 remained normal. Apparently the air in the cases where metallic mercury or bichloride had been used was contaminated with some toxic substance. In one case the poisonous ingredient was known to be mercury vapor, and since the injury in the two cases was similar the assumption was that bichloride in the soil had been reduced to mercury, thus affording a source for metallic mercury vapor. A report setting forth this view was published in May, 1933 (6). The early assumptions have been well supported by results of additional experiments with these two chemicals. Mercury has been recovered from leaves exposed to vapors emanating from soil treated with bichloride and metallic mercury vapor has been detected in the air surrounding treated

soil. The amount of injury to leaves at any time was correlated with the vapor pressure of mercury which in turn was governed by temperature. Also, 13 other compounds of mercury, when used the same as bichloride to moisten soil, caused similar injury to leaves, and many different species of plants have been found sensitive to the vapors.

The report in this paper shows in some detail the results of the experiments performed to determine the effect of vapors of mercury and its compounds on growing plants when exposed under various conditions.

MATERIALS AND METHODS

In the first experiment four varieties of two-year-old rose plants growing in a greenhouse according to commercial methods were exposed to vapors emanating from rich soil treated with bichloride of mercury. During the test the ventilators were regulated as usual in rose growing with no special effort to hold the vapors in the house. In all other experiments potted plants which could be placed in glass cases or under bell jars were used. The cases were built of window sash on a board bottom which was covered with galvanized metal. When completed, the cases measured 4 feet x 3 feet x 3 feet with a door 15 inches x 24 inches. The joints were sealed on the inside with modeling clay, except the doors which were sealed from the outside after the experiments were started. When single plants were used they were placed on glass plates together with the material to which they were to be exposed and covered with bell jars.

To expose a plant to mercury vapor a beaker or pan of the metal was enclosed with the plants. The volume was not important, but the total surface exposed determined the rate of injury with a given time and temperature. For example, one cubic centimeter broken into small globules was more effective than the same volume in one mass.

Compounds of mercury were tested as solutions or dry crystals and mixed with soil, sand, and tankage. Plants were enclosed in cases or bell jars with various amounts of the pure chemicals in Pyrex beakers or sprayed on glass plates. To determine the effect of vapors from soil or tankage when mixed with the chemicals a quart of the material moistened with 0.05 per cent (or stronger) solution of the compounds was sufficient for one of the large cases. Under bell jars 100 cc. of the soil were sufficient. The minimum amount required was not determined, but the results varied with the amount of organic matter present in the soil. Consistent results were obtained when tankage was used because it was consistently high in organic matter. Sand and sandy soil were much less effective than tankage.

The temperature effects were obtained by conducting the experiment with plants under bell jars in dark constant temperature rooms held at 35°, 40°, 50°, 60°, and 73° F. The temperature in the cases varied with the greenhouse air and light intensity.

Other details of methods will be given when necessary under "Experimental Results."

EXPERIMENTAL RESULTS

A total of 75 genera have been exposed to vapors from mercury compounds. Of this number aloe (*Aloe arborescens* Mill.), croton (*Codiaeum* sp.), English ivy (*Hedera helix* L.), Jerusalem cherry (*Solanum pseudocapsicum* L.), oak (*Quercus* sp.), orchid (*Laeliocattleya* sp.), pachysandra (*Pachysandra terminalis* Sieb. & Zucc.), pandanus (*Pandanus veitchi* Dall.), sarcococca (*Sarcococca pruniformis* Lindl.), and tobacco (*Nicotiana tabacum* L.) were found comparatively resistant in part or over the entire plant. The following 65 species were susceptible to injury: abutilon (*Abutilon hybridum* Voss.), columbine (*Aquilegia* sp.), azalea (*Azalea obtusum* Planck var. *amoenum* Rehd.), balsam (*Impatiens balsamina* L. vars. Pure White and Shining Scarlet), barley (*Hordeum vulgare* L.), basswood tree (*Tilia americana* L.), bayberry (*Myrica carolinensis* L.), bean (*Phaseolus* sp. and *Vicia faba* L.), begonia (*Begonia semperflorens* Link & Otto var. Vernon), bryophyllum (*Bryophyllum pinnatum* Kurz.), butterfly weed (*Asclepias tuberosa* L.), camellia (*Camellia japonica* L. var. Dixie), carnation (*Dianthus* sp.), endive (*Chicorium endivia* L.), cinquefoil (*Potentilla reptans* L.), coleus (*Coleus blumei* Benth.), cosmos (*Cosmos sulphureus* Cav.), coto-neaster (*Cotoneaster horizontalis* Desne.), crab grass (*Digitaria sanguinalis* [L.] Scop.), eggplant (*Solanum melongena* L. var. *esculentum* Nees.), fern (*Nephrolepis exaltata* Scott var. *bostoniensis* Davenport and *Cyrtomium falcatum* Presl.), forsythia (*Forsythia intermedia* Zabel), fuchsia (*Fuchsia hybrida* Voss), gardenia (*Gardenia jasminoides* Ellis), geranium (*Pelargonium hortorum* Bailey), grape (*Vitis labruscana* Bailey var. Concord), holly (*Ilex opaca* Ait. and *Ilex cornuta* Lindl.), honeysuckle (*Lonicera* sp.), hydrangea (*Hydrangea macrophylla* DC.), lily (*Lilium longiflorum* var. Erabu), calla lily (*Zantedeschia aethiopica* Spreng.), Japanese maple (*Acer palmatum* Thunb.), marigold (*Tagetes erecta* L.), mimosa (*Mimosa pudica* L.), mustard (*Brassica nigra* Koch and *Brassica alba* Rabenh.), oxalis (*Oxalis corniculata* L. var. *repens* Zucc.), pea (*Pisum sativum* L.), peach (*Prunus persica* [L.] Stokes), persimmon (*Diospyros virginiana* L.), white pine (*Pinus strobus* L.), privet (*Ligustrum ovalifolium* Hassk.), radish (*Raphanus sativus* L.), rhodotypos (*Rhodotypos kerrioides* Sieb. & Zucc.), rose (*Rosa* [hybrid tea] vars. Briarcliff, Columbia, Coolidge, Killarney, Madame Butterfly, Pernet, Premier Supreme, Talisman, Templar), rye (*Secale cereale* L.), salsify (*Scorzonera hispanica* L.), salvia (*Salvia splendens* Ker.), saxifrage (*Saxifraga parmentosa* L.), silver bell (*Halesia carolina* L.), strawberry (*Fragaria chiloensis* Duchesne), sunflower (*Helianthus debilis* Nutt.), timothy (*Phleum pratense* L.), tomato (*Lycopersicon esculentum* Mill. vars. Bonny Best, Marglobe, Magnus), tree of heaven (*Ailan-*

thus *glandulosa* Desf.), tree peony (*Paeonia suffruticosa* Andr.), viburnum (*Viburnum dentatum* L. and *Viburnum opulus* L.), vinca (*Vinca major* L.), wandering Jew (*Zebrina pendula* Schnizl.), wheat (*Triticum aestivum* L.), willow (*Salix babylonica* L.).

Leaves and floral organs were most susceptible to attack from vapors of mercury or compounds of mercury. Stems and leaf buds were not injured unless the temperature was high and the treatment severe as to duration and concentration. Injury was never uniform over the entire plant, the old leaves usually being more susceptible than young while young flower buds usually showed more injury than the old. Details concerning injury of the different species will be given in the various sections to follow.

ROSES

Grafted plants of Briarcliff and Premier Supreme roses were prepared for treatment by being grown in two-gallon jars with fertile soil until they were in an active state of growth and beginning to form many new flower buds. Twenty-eight plants of fairly uniform size were divided into two lots of 14 each (ten Briarcliff and four Supreme) and placed across a narrow aisle from each other in a greenhouse. Each plant of one set was watered at three different intervals with a quart measure full of either 0.2 per cent or 0.05 per cent bichloride of mercury solution. The controls were given only tap water. The plants appeared normal during the first two weeks but thereafter the flowers of both sets lacked the bright pink pigment characteristic of the Briarcliff and Premier Supreme varieties. The injury increased as time went on and after 21 days the flowers were either badly faded or brown, though the leaves did not appear to be injured.

At the beginning of the fourth week a muslin cage was built around the 14 treated plants together with an equal number of new specimens as controls. The cage was opened only when the plants were watered or bichloride was added to the soil of the treated set. The injured buds were removed from the control plants on the opposite bench, but they were left in the same position as before.

In the muslin cage the 14 plants previously described showed increased injury and the new controls developed flowers with the characteristic faded pink and brown petals. Many of the buds failed to unfold and the corollas abscissed from the receptacle. The stamens of injured buds usually varied in color from dark brown to nearly black, differing from the light brown color of the normal controls (Fig. 1 A). The plants growing in soil to which bichloride had been added were not more severely injured than others within the muslin cage. Those on the opposite bench which had been previously injured before the treated plants were enclosed in the muslin cage recovered and produced approximately normal flowers after 21 days. These results indicated rather conclusively that the bichloride was in

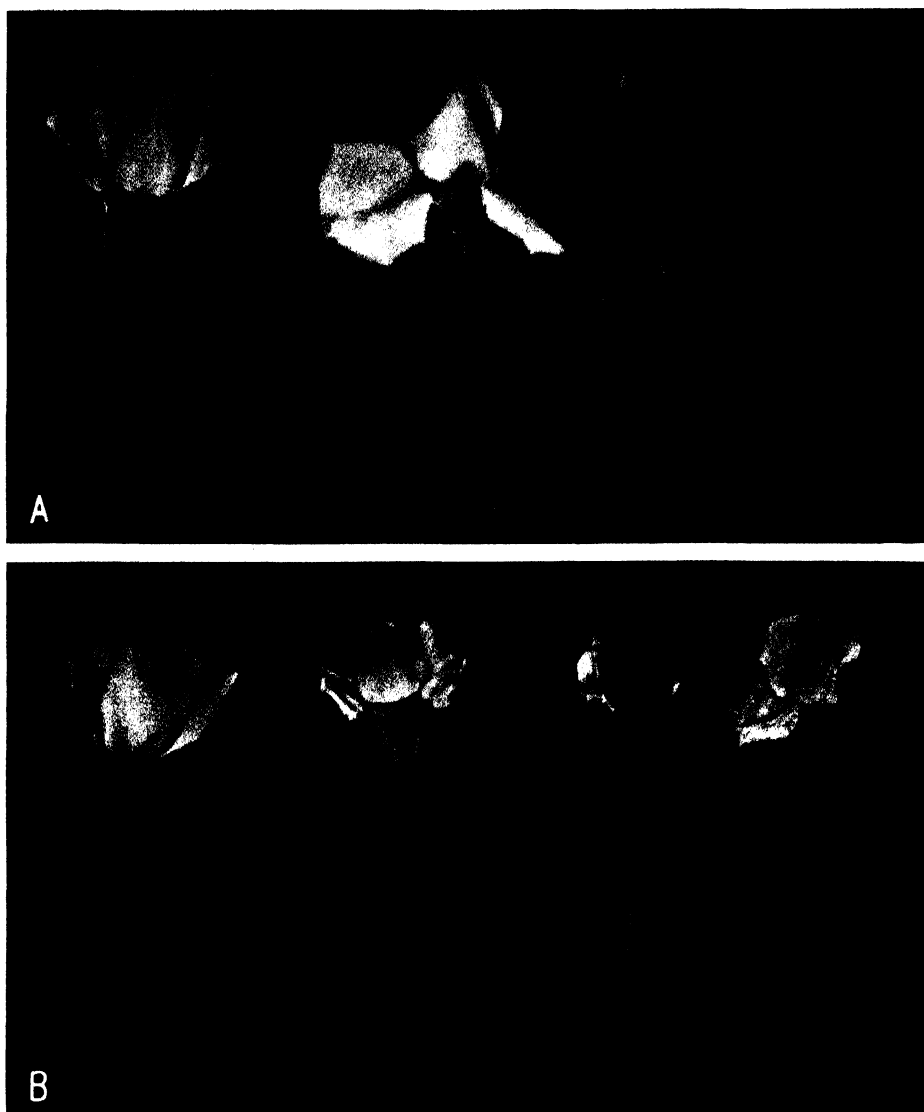


FIGURE 1. Briarcliff rose buds. A. Left to right: 1, normal control bud; 2, bud from plant in muslin cage where 14 others had been watered with 0.05 per cent mercuric chloride; 3 and 4, buds from plants in muslin cage watered with 0.05 per cent mercuric chloride. B. Left to right: 1, normal bud from control glass case; 2, 3, and 4, buds from plants in glass case where three others had been watered with 0.05 per cent mercuric chloride.

some way responsible for the injury, first to plants on the open bench and then within the cage. Whether the bichloride as such had gone into the air to injure the roses or whether it was first reduced to the metallic state could not be determined from the information at hand. In order to obtain more conclusive data six potted roses were placed in each of three glass cases and treated as follows: Case No. 1, used as control; Case No. 2, three plants watered with 0.05 per cent bichloride and the other three with water; Case No. 3, 500 cc. of metallic mercury in an open granite pan enclosed in the case with the rose plants. The temperature within the cases was approximately 70° F. during the night, but rose to 90° F. or more during the time the sun shone directly on the cases.

The control plants in Case No. 1 remained normal throughout the experiment; those in Case No. 2 where bichloride was applied had injured leaves in four days and bud injury in six days. All six plants were equally affected though only three had been treated with the mercury compound (Fig. 1 B). In the third case where the plants had been exposed to metallic mercury, leaf injury occurred within 48 hours. Flower buds were injured in patches over the sepals and the petals which developed later lacked the pink color which is characteristic for Briarcliff. After six days of exposure the plants were placed out on an open bench in the greenhouse. Most of the leaves dried and fell from the plants. Young buds were severely injured and ten days from the time the experiment was started peduncles of young flower buds turned dark brown as shown in Figure 1 B where bichloride was used. In general, the injury caused by metallic mercury vapor was indistinguishable from that induced by vapors of soil treated with bichloride. Also, recovery was about the same for both sets. An abnormally large number of new shoots arose promiscuously over the stems and produced flowers with normal color.

A total of 14 tests involving 398 rose plants were made during the course of these experiments. The results were similar throughout and details, therefore, may be omitted. One phase of the work concerning varietal difference, however, deserves special mention. In the first greenhouse experiment there were four beds, each containing equal numbers of four varieties, Madame Butterfly, Briarcliff, Templar, and Mrs. Calvin Coolidge. One bed was treated with bichloride of mercury solution poured over the soil. Three weeks later the majority of flowers of Briarcliff over the entire range lacked the bright pink pigment normal for the variety and many petals were brown. Madame Butterfly and Mrs. Calvin Coolidge varieties were slightly faded but were not browned. Templar did not appear to be affected. There was no evident leaf injury. Apparently, therefore, with low concentrations the floral parts are more sensitive than leaves, and of the four varieties, Briarcliff appeared the most sensitive.

The following varieties were exposed in glass cases to vapors emanating

from a quart of tankage moistened with one per cent bichloride of mercury solutions: Columbia, Templar, Killarney, Pernet, Butterfly, Coolidge, and Talisman. Under the conditions of these experiments flower buds and leaves of all seven varieties were severely injured. Pernet seemed to be slightly more resistant than others, though the difference was not striking. From appearances alone there was, however, no good basis for comparison. Neither do chemical analyses, given in another section of this paper, help to account for the varietal differences reported for low dosages of mercury in the greenhouse tests, in which cases floral parts of Briarcliff were more sensitive than three other varieties.

Ratzek (5) reported injury to Briarcliff roses in an experimental house at Cornell University after some of the beds had been treated with bichloride to kill earthworms. A difference in susceptibility of varieties in the house was noted. He later induced the same type of injury experimentally with metallic mercury and bichloride of mercury and again found Briarcliff one of the most sensitive varieties.

In 1797 there was made known in a letter addressed to Van Mons by Lauwerenburgh that four Dutch chemists, Deiman, Paats, Van-Troostwyck, and Lauwerenburgh (3) had discovered the deleterious effects of metallic mercury vapors on plants. The results of 15 experiments mentioned in the letter, showed that where beans, mints, or spiraea were enclosed in bell jars with metallic mercury the leaves became spotted after 24 hours and if left exposed to the vapors for several days the plants died. In 1867 Boussingault (2) repeated some of the experiments, obtaining results comparable to those of the Dutch scientists. Since that time several German workers have discussed the poisonous effects of metallic mercury vapors, but no publications have been found which show that the air becomes contaminated where mercuric compounds are applied to the soil.

PEACH SEEDLINGS

Peach seedlings were found very susceptible to attack from vapors of metallic mercury as well as vapors emanating from organic matter treated with bichloride of mercury. The first signs of injury occurred on old leaves within 48 hours after the plants were placed in a glass case with metallic mercury or compounds of mercury mixed with soil. Usually the interveinous tissues faded slightly and then turned brown. If the injury was severe, the whole leaf turned brown. With milder dosages many spots appeared first on the oldest leaves and then more generally over the plant, the youngest leaves being the most resistant. The leaves were seldom uniformly browned unless the temperature was from 75° to 80° F. or higher. Abscission occurred especially where the injury came on gradually. At high temperatures the injury was rapid and abscission sometimes did not occur.

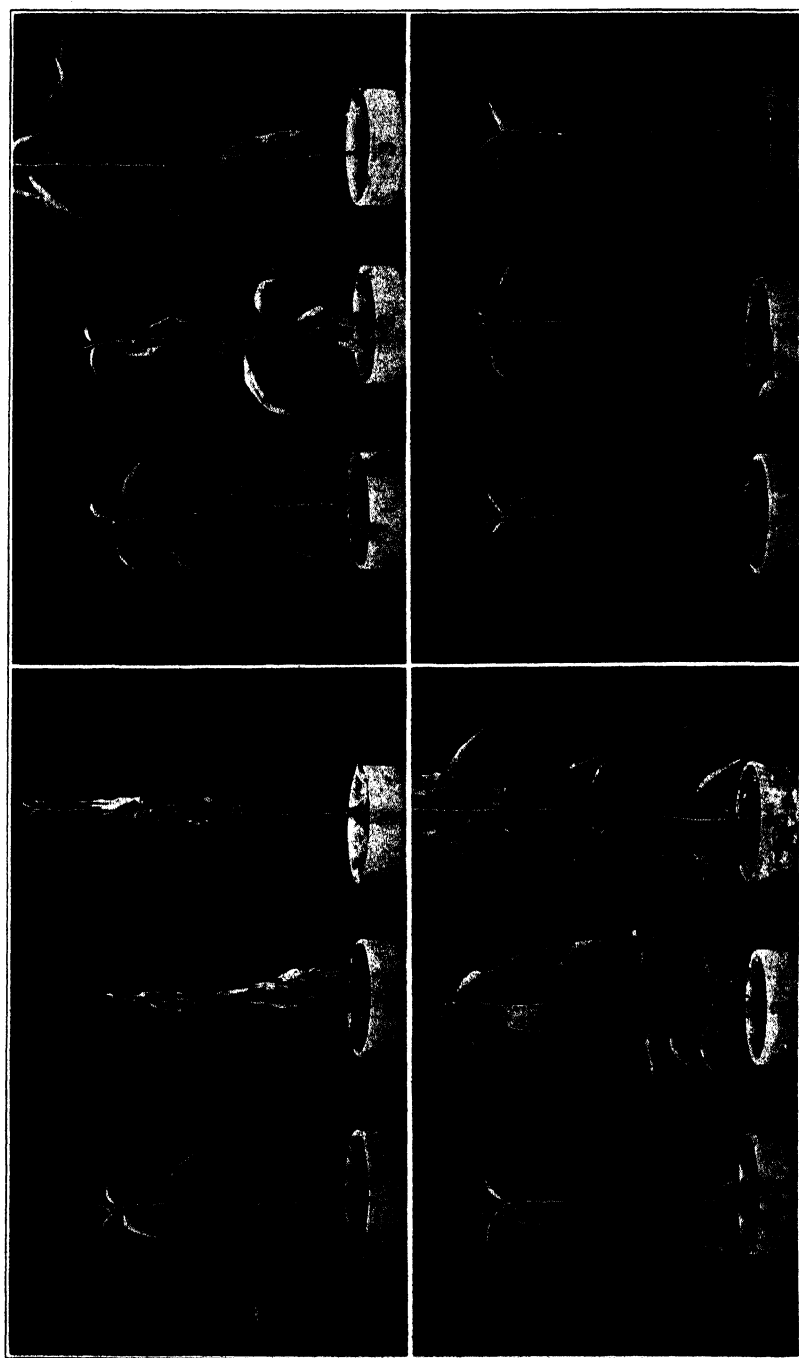


FIGURE 2. Effect of temperature on peach seedlings confined five days under bell jars as follows: 1, control; 2, with 400 cc. of tankage moistened with one per cent mercuric chloride solution; 3, with 5 cc. of metallic mercury in open beaker.

Stem and bud tissues were more resistant than leaves. Plants treated until entirely defoliated made complete recovery after being removed to normal conditions. When the treatment lasted five days or more, thereby killing all leaves, the stems also were injured either uniformly or over patches of bark. In some cases the stem and buds were killed from the tip down for a distance of six inches or more and still the rest of the stem appeared normal and produced new shoots.

The type of injury on leaves from vapors of metallic mercury was indistinguishable from that resulting from treatment with organic matter wet with bichloride of mercury (Fig. 2 A and B). Both caused browning or spotting of old leaves first, then young, and later promiscuous abscission.

PRIVET

The first evidence of injury to privet plants from exposure to vapors of mercury or compounds of mercury was shown by slight browning of inter-veinous portions and progressive abscission beginning with the oldest leaves. Frequently green leaves fell without signs of injury on the blade, indicating that the abscission layer is more susceptible to attack than other tissues. The actively growing tips were very resistant as compared with peach seedlings. When removed from the toxic vapors after five days of exposure, privet plants made a quick recovery, tips continuing to grow and many new shoots appearing.

RESPONSES OF OTHER SPECIES

The responses of rose, peach, and privet plants to mercury vapors were described in some detail. Collectively these three types characterize responses made by susceptible species in general. The species were not equally sensitive. For example, butterfly weed and broad bean in a case with a quart of tankage moistened with bichloride solution were severely injured in less than 24 hours, whereas tobacco plants in the same case showed only slight injury on old leaves after 48 hours and the young leaves tolerated the treatment for 168 hours without visible injury.

Forty-two different species of plants were placed in a large glass case with a quart of tankage wet with a one per cent solution of bichloride so that differences in sensitivity of species could be studied. The temperature in the case varied from 65° F. at night to 98° F. in daylight while the sun was shining. The average temperature for the entire time was probably above 70° F. though no attempt was made to record hourly temperatures. The results showing the estimated amount of injury at four different times are recorded in Table I. Croton, aloe, and sarcococca withstood the entire period of exposure without showing injury. Two weeks after removal from the case these three plants developed a few spots on the leaves and the

croton plant lost three of its older leaves, showing that though comparatively resistant these species were slightly injured.

TABLE I

COMPARATIVE INJURY TO PLANTS CAUSED BY VAPORS EMANATING FROM FERTILE SOIL WHICH HAD BEEN MOISTENED WITH ONE PER CENT BICHLORIDE OF MERCURY

Plants	Injury occurring after various hours of treatment			
	24	48	72	168
Aloe	o	o	o	o
Azalea	o	x	x	xx
Basswood seedling	o	x	xx	xxx
Bean (broad)	xxx	xxx	xxx	xxxx
Begonia	o	o	xx	xx
Butterfly weed	xxx	xxx	xxx	xxxx
Camellia	o	o	x	xx
Cinquefoil	x	xx	xxx	xxxx
Columbine	o	o	x	x
Cosmos	o	x	xx	xxx
Cotoneaster	o	x	x	xxx
Croton	o	o	o	o
Fern (Boston)	x	xx	xxx	xxx
Fern (holly)	o	xx	xxx	xxx
Forsythia	o	x	xx	xxx
Fuchsia	o	x	xx	xx
Geranium	o	x	xx	xxx
Holly (American)	o	o	x	xxx
Holly (Chinese)	o	o	o	x
Hydrangea	o	xx	xxx	xxx
Ivy	o	o	o	?
Jerusalem cherry	o	o	o	x
Lily (calla)	o	x	x	xxx
Lily (Easter)	o	o	xx	xxx
Maple (Japanese)	o	o	x	xx
Mimosa	o	xxx	xxx	xxx
Oak	o	o	x	x
Oxalis	xxx	xxx	xxx	xxx
Peach	o	x	xx	xxx
Persimmon	o	x	xx	xxx
Pine (white)	o	o	x	xx
Privet	x	xx	xx	xxx
Salvia	o	x	xx	xx
Sarcococca	o	o	o	o
Saxifrage	o	o	x	xx
Strawberry	o	o	x	xx
Sunflower	xxx	xxx	xxx	xxx
Tobacco	o	x	xx	xx
Tomato	x	x	xx	xxx
Viburnum	o	x	xx	xxx
Vinca	o	x	xxx	xxx
Willow	x	xx	xxx	xxx

o = no apparent injury; x = slight injury on a few leaves; xx = approximately 25 per cent of leaves injured; xxx = 50 per cent or more of leaves injured; xxxx = all leaves injured.

Unfortunately these 42 species were not all in the same stage of growth, some being active and others approaching or in dormancy. To make a fair comparison all should be active or dormant. For the most part the growing

tips with their youngest leaves were not injured within a period of five days. Tomato plants continued to elongate throughout the time they were being exposed though the mature leaves were killed. Mature fern leaves also were injured in 48 hours while young fronds of the same plant toler-

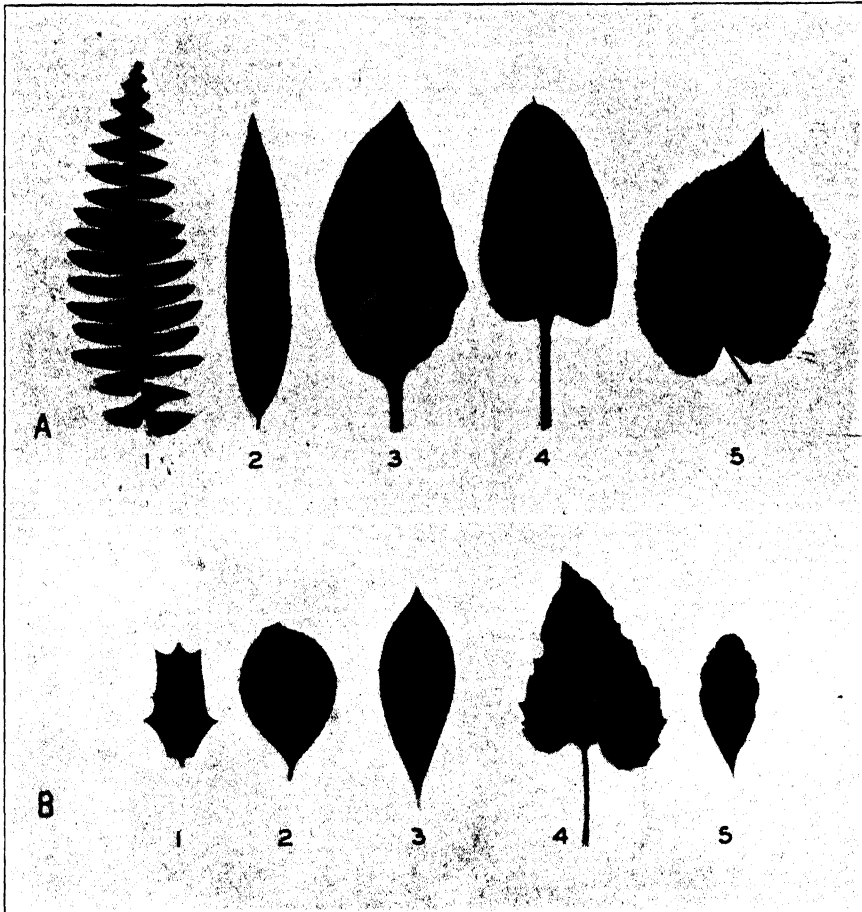


FIGURE 3. Type of injury on leaves from ten different species confined in a glass case for five days with two liters of tankage moistened with one per cent mercuric chloride solution. A. 1, fern; 2, peach; 3, tobacco; 4, calla lily; 5, basswood. B. 1, holly; 2, camellia; 3, persimmon; 4, sunflower; 5, bayberry.

ated five days of treatment without visible injury. Leaflets of half-mature fronds were injured at the base of the blade, indicating an age difference between that region and the rest of the leaf. The interveinous portions of tobacco leaves were killed while the veins and petioles appeared not to be injured. Peach leaves were usually spotted promiscuously and often

injured along the mid-veins. The blades were never uniformly injured in any of the species unless the temperature and dosage were both high. Figure 3 shows the variation which might be expected among species if the plants are all exposed to the same toxic vapors. The fern frond is especially interesting because it shows definitely that the degree of injury varies with the age of the tissue.

EFFECT OF TEMPERATURE ON RATE OF INJURY

To determine the effects of various temperatures on rate of injury from mercury vapors, 15 peach seedlings were enclosed in bell jars and then divided into five lots of three plants each to be placed in dark storage rooms at the following constant temperatures: 75°, 60°, 50°, 40°, and 35° F. Of each set one plant was used as a control, one was enclosed with 400 cc. of tankage moistened in a beaker with 200 cc. of one per cent mercuric chloride, and the third was enclosed with 5 cc. of metallic mercury in a 100 cc. beaker. The plants were inspected daily without being removed from the bell jars. After five days they were all removed, placed in a large glass case for 24 hours, and then photographed (Fig. 2). The first visible injury was recorded for the lot at 75° F. after 48 hours of exposure. The experimental plants at this high temperature were injured first on the basal leaves. By the fourth day all of the leaves were injured. No definite injury could be detected on plants of the other lots until after removal from the bell jars. Upon close inspection, however, there was some indication of injury to the leaves of the lots at 60° and 50° F. After standing at higher temperature in a glass case for 24 hours the injury was evident. The lots at 40° and 35° F. were not injured. All leaves and the stems of the plants treated at 75° F. were killed but plants of all other lots recovered and two months later appeared normal except for the absence of some leaves of those that were treated at 60° and 50° F.

These results show that the degree of injury was in proportion to the different temperatures maintained, decreasing with decreasing temperature to 40° F. where there was no evident injury. The degree of injury appears to be correlated also with vapor pressure of mercury at these different temperatures (Fig. 4).

COMPARATIVE INJURY FROM 14 DIFFERENT MERCURY COMPOUNDS

Plants were exposed to vapors emanating from soil moistened with solutions of 14 different mercury compounds. Eight of these were inorganic and six organic as follows: mercuric bromide, mercuric chloride, mercuric cyanide, mercuric iodide, mercuric nitrate, mercuric oxide, mercuric sulphate, mercurous chloride, Uspulun (Bayer Co.), Semesan (Bayer-Semesan Co.), Nu-Green (Bayer Co.), Dubay 1101T (four per cent ethyl mer-

cury arsenate), Dipdust (Bayer Co.), and Mercurochrome. Two grams of each compound were mixed with 400 cc. of moist, fertile soil in a Pyrex beaker and placed with the plants to be tested under 18-liter bell jars at room temperature which fluctuated within a few degrees around 70° F. The data from the experiment with peach seedlings are shown in Table II. It will be seen that in every case the vapors arising from the treated soil were toxic to the plants. In another experiment these same chemicals were mixed with moist peat moss and tested on privet as described above for

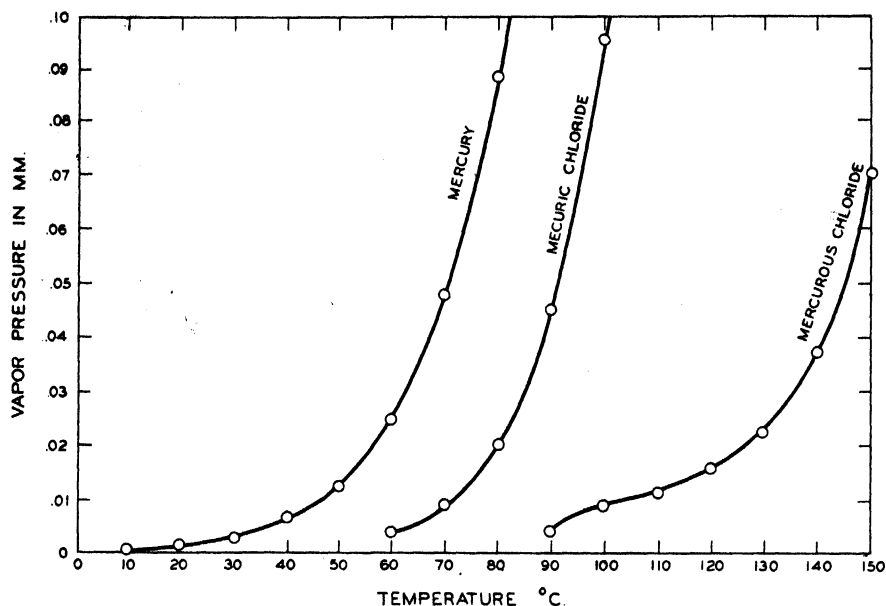


FIGURE 4. Curves showing the relationships of the vapor pressures of metallic mercury, mercuric chloride, and mercurous chloride at different temperatures. All data taken from International Critical Tables.

peach seedlings. The privets were injured in every case and, as with the peach seedling, the type of injury resembled that produced by metallic mercury. The results suggest, that, through reduction of the compounds, metallic mercury was liberated and the vapors attacked the plants. This does not, however, preclude the possibility that vapors from the compounds themselves play a part, especially those that have considerable vapor pressure at room temperature.

In connection with the experiments for testing with the various compounds of mercury it was noted that where soil was heavily fertilized with tankage and then treated with the chemicals, fungi grew luxuriantly. Though the vapors emanating from the medium injured green plants, these

TABLE II

COMPARATIVE INJURY TO PEACH SEEDLINGS FROM 13 DIFFERENT MERCURY COMPOUNDS AFTER TWO GRAMS OF THE CHEMICAL WERE MIXED WITH 400 CC. OF MOIST FERTILE SOIL IN A BEAKER AND PLACED WITH PLANTS UNDER BELL JARS AT 70° F. OR HIGHER IN THE LABORATORY

Chemical	Total no. on leaves on plants	No. of leaves injured after various days of treatment				No. of leaves abscised after 7 days
		2	3	4	7	
Control	35	0	0	0	0	0
Dubay	55	0	0	2	15	3
Mercuric bromide	34	0	0	7	34	21
Mercuric chloride	46	2	5	46		46
Mercuric cyanide	43	0	0	22	43	21
Mercuric iodide	60	0	0	10	60	20
Mercuric nitrate	38	4	31	38		38
Mercuric oxide	78	8	68	75	78	68
Mercuric sulphate	66	6	7	30	66	66
Mercurous chloride	56	56	56			56
Metallic mercury	26	26				26
Nu-Green	40	0	2	2	40	31
Semesan	35	—	—	35		35
Uspulun	28	10	28			28

fungi were very tolerant. Table III shows the genera identified after various treatments.

TABLE III

FUNGI FOUND GROWING ON FERTILIZED SOIL SEVEN DAYS AFTER IT HAD BEEN TREATED WITH A ONE PER CENT SOLUTION OF DIFFERENT MERCURY COMPOUNDS

Chemicals	Fungi			
	<i>Penicillium</i>	<i>Mucor</i>	<i>Aspergillus</i>	<i>Botrytis</i>
Mercuric sulphate	x	x		
Mercuric oxide	x			
Mercuric iodide	x	x		
Mercuric chloride	x	x	x	x
Mercuric cyanide				
Mercuric bromide	Uncertain			
Mercuric nitrate	x			
Mercurous chloride	x			
Nu-Green	x	x	x	
Dipdust	x	x		x
Uspulun	x			
Semesan	x	x		
Dubay				
Mercurochrome	x	x		
Check		x		

x indicates presence of fungus.

DETERMINATION OF MERCURY IN LEAVES

When plants were enclosed in a glass case with soil which had been moistened with a solution of bichloride of mercury, the leaves were defi-

nately injured. The question then arose as to whether or not mercury could be recovered in the injured parts. The amount of mercury, if any, would necessarily be small because only small amounts were added to the soil in the beginning. Since an exact quantitative method for determining small amounts was essential, the precipitation method of the Association of Official Agricultural Chemists was used (1, p. 64).

One important modification was made in the method; instead of the Erlenmeyer flask with an air condenser, the Kjeldahl flask was used. This change was made because an 8 g. sample of dry leaves was hard to digest with the air condenser. Also, by tests where known amounts of HgCl_2 were added to 8 g. of dry leaves, more mercury could be recovered by using Kjeldahl flasks for digestion than by the use of flasks with air condensers.

At the beginning 20 cc. of superoxol were added to each 8 g. sample after the leaves had been mixed in the Kjeldahl flask with 75 cc. of H_2SO_4 . Thereafter the superoxol was added at the rate of 10 cc. at intervals until 30 to 40 had been used, depending upon the time required to digest the organic material. Heating was stopped as soon as all the dark particles disappeared though the solution was yellow instead of white. This was thought advisable so as not to drive off the mercury. The Official method calls for 0.1 N potassium permanganate solution for titration to destroy excess hydrogen peroxide. This was modified to 0.01 N potassium permanganate because titration indicated practically no hydrogen peroxide present after digestion. The black precipitate obtained by this method of analysis was mercuric sulphide, 0.86219 of which was calculated as mercury.

To test the efficiency of the methods known amounts of mercuric chloride were added to dry peach leaves and then attempts were made to recover the mercury. The results are shown in Table IV.

TABLE IV
MERCURY RECOVERED FROM AN EIGHT-GRAM SAMPLE OF DRY PEACH LEAVES TO WHICH KNOWN AMOUNTS OF BICHLORIDE OF MERCURY HAD BEEN ADDED

Amount of bichloride added	Calculated as mercury in mg.	Mercury recovered in mg.
2 cc. of 1.0%	14.76	12.07
2 cc. of 0.5%	7.38	7.24
2 cc. of 0.25%	3.69	4.57

Eight grams of oven-dry leaves from treated plants, usually taken as the sample to be analyzed, were compared with an equal amount from control leaves. In every case mercury was recovered from the leaves of treated plants. The exact amounts of mercury found are shown in Table V. The difference between the samples from treated and control plants could be readily observed without weighing due to the black precipitate in the

former at the time hydrogen sulphide was passed through the solutions. Also when filtered through the Gooch crucible, the treated caused the asbestos pads to become black, while those of the controls remained almost colorless. The weight of the precipitate of the control was subtracted from that of the treated sample before the total mercury was calculated.

TABLE V

CHEMICAL ANALYSES OF EIGHT-GRAM SAMPLES OF DRIED LEAVES FROM PLANTS EXPOSED TO FERTILIZED SOIL MOISTENED WITH BICHLORIDE OF MERCURY

Plants	In glass cases or bell jars for various time periods	Determinations		
		Weight of precipitate in mg.	Total mercury in mg.	P.p.m. of dry weight
Briarcliff rose	Treated 4 days Control 4 days	4.1 1.15	2.54	317
Killarney rose	Treated 4 days Control 4 days	5.7 0.0	4.92	615
Coolidge rose	Treated 4 days Control 4 days	8.01 0.5	6.47	808
Peach	Treated 3 days in dark	5.5	3.79	473
	Treated 3 days in light	5.6	3.88	485
	Treated 5 days in dark	7.0	5.08	635
	Treated 5 days in light	6.9	5.0	625
	Control 5 days in light	1.1		
Jerusalem cherry	Treated 4 days Control 4 days	12.9 0.7	10.51	1313
Tomato	Treated 51 hours Control 51 hours	11.2 1.1	8.7	1087
Rhodotypos	Treated 8 days Control 8 days	5.0 1.3	3.19	398
Silver bell	Treated 8 days Control 8 days	8.2 0.7	6.46	807
Gardenia	Treated 8 days (injured)	8.15	7.03	878
	Treated 8 days (not injured)*	11.19	10.26	1282
	Control 8 days	0.0		
Tobacco (tip leaves)	Treated 7 days Control 7 days	15.0 0.3	12.67	2405
Tobacco (middle leaves)	Treated 7 days Control 7 days	24.6 0.17	19.74	3747
Tobacco (basal leaves)	Treated 7 days Control 7 days	21.8 3.2	16.03	3044

* Leaves did not appear to be injured at time they were collected, but comparable leaves left on the plants, though not further exposed to the treated soil, later showed considerable injury.

VAPORS EMANATING FROM TREATED SOIL OR ORGANIC MATTER

Vapor arising from metallic mercury was definitely toxic to plants. The injury it caused resembled that resulting from vapors emanating from soil treated with any of 13 different mercury compounds. The question which naturally arose was whether the compounds were first reduced by the soil to the metallic state or whether vapors from the compounds themselves caused the injury. The chemical analyses definitely showed that mercury could be recovered from leaves of plants exposed to soil which

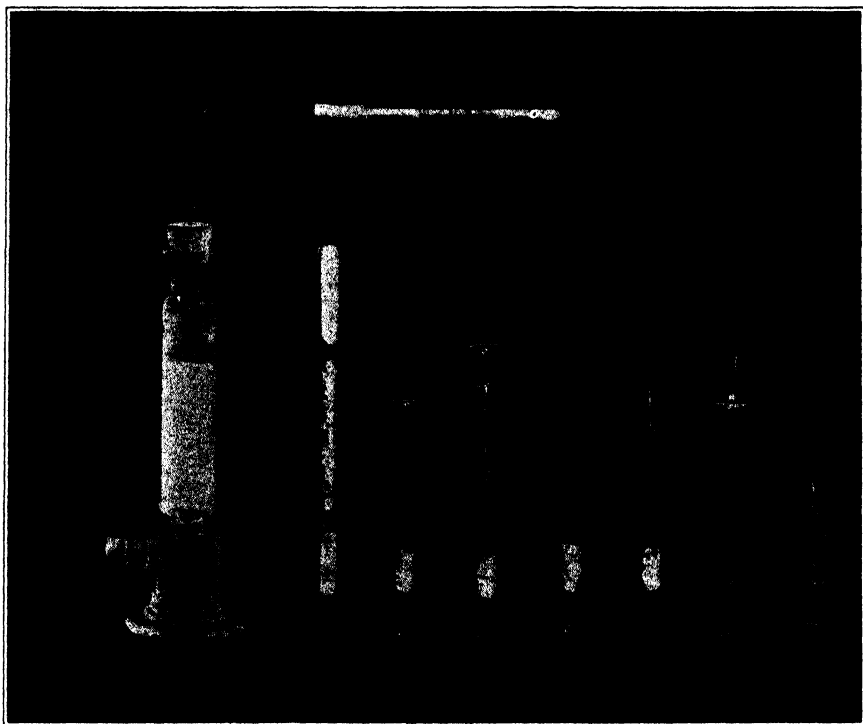


FIGURE 5. Apparatus and method used for detecting metallic mercury vapor in soil treated with mercury compounds. A. Absorption tower filled with treated soil or other material through which air could be drawn and thence over gold leaf to permit of the formation of mercury-gold amalgam. B. Steps in method for detecting amalgam: 1, gold leaf after treatment in "A"; 2, gold leaf removed to test tube; 3, capillary made; 4, heated gold leaf; 5, removal of gold; 6, upper portion of tube placed over a crystal of iodine; 7, iodine vapor reacted with mercury condensed on the wall of the tube forming bright red mercuric iodide.

had been moistened with a bichloride solution. There was no indication, however, as to the form of mercury which passed into the leaves. If metallic mercury vapor could be detected in the atmosphere about the plant, this fact would be supporting evidence for the theory that at least some of the

compound when added to soil was reduced to the metallic state. To test this possibility air was drawn through fertile soil treated with bichloride and then over gold leaf confined in a glass tube so that if any mercury vapor were present, it could be amalgamated with the gold. The apparatus used for this purpose is shown in Figure 5 A. After air had been drawn through the soil and over the gold leaf for several hours it was necessary to find if mercury had actually been amalgamated with the gold. The gold leaf was, therefore, transferred to the bottom of a small test tube, which was drawn out to a capillary near the top. Heat was applied to the gold leaf to drive off mercury which, if present, condensed on the wall of the test tube just back of the capillary. The bottom of the tube was then cut off, the gold removed, and the shell placed in a slightly larger size test tube over a crystal of iodine. The inside wall of the capillary tube was in this way exposed to vapors of iodine and if any mercury was present, mercuric iodide was formed. The method used is shown step by step in Figure 5 B. The brilliant red color of mercuric iodide made possible the detection of small amounts of condensed mercury vapor on the wall of the glass tube. The color could be seen easily when the amount of condensed mercury was so small it could not be detected with the naked eye. With this simple method air from a number of sources was tested for the presence of metallic mercury vapor. Positive results were obtained under the following conditions where bichloride of mercury had been used: (a) air drawn for ten hours through an absorption tower full of treated fertile soil; (b) air drawn for ten hours through an absorption tower full of treated tankage; (c) air drawn through treated tankage any time within four months after treatment; (d) air drawn through a bell jar containing a 400 cc. beaker of treated soil, then over a plant in a second bell jar, finally passing over gold leaf at the exit (Fig. 6); (e) air drawn through a bell jar containing a plant together with a glass plate previously atomized with a solution of bichloride and then dried.

Negative results were obtained under the following conditions: (a) air drawn for 10 hours through an absorption tower full of mercuric chloride crystals and then over gold leaf; (b) air drawn for ten hours through a bell jar containing only a glass plate atomized with bichloride solution (compare with "e" above).

Similar results were obtained wherever mercurous chloride was substituted for mercuric chloride except "e" listed under "positive results." When mercurous chloride was mixed with wet soil and enclosed with a plant the leaves showed injury in 24 hours, but when sprayed on a glass plate and enclosed with a plant it did not cause injury. Mercuric chloride sprayed on a glass plate and enclosed with a plant was very effective.

Both organic mercury and inorganic mercury compounds were similarly reduced when placed in contact with organic matter. When air was

drawn for 72 hours or more over tankage treated with mercuric cyanide, Semesan, Dipdust, or Nu-Green and then over gold leaf, the amalgam was so great that the gold leaf turned from gold to mercury color. Vapors emanating from fertile soil treated with these chemicals injured plants badly within 48 hours in a temperature of 75° F. or above.



FIGURE 6. Apparatus used to determine the effect of vapors emanating from soil treated with mercury compounds and to detect the presence of metallic mercury vapor by use of gold leaf at the exit on the right.

DISCUSSION OF RESULTS

With any given set of conditions the degree of injury caused by metallic mercury vapors varied with the temperature, the amount of metallic surface exposed to the air, and the length of time plants were subjected to the vapor treatment. When hot mercury was placed in a glass case at room temperature with rose plants, all the leaves were killed within a few hours. The same volume of mercury at 70° F. with the same surface exposed required more than 48 hours to kill all the leaves. One cubic centimeter of finely divided mercury was more effective than 100 cc. with a small surface exposed to the air. The degree of injury depended, therefore, upon the concentration of the mercury vapor in the air surrounding the plant. Any treatment which prevented vaporization of mercury under the bell jar

protected the plant, as, for example, a thin film of water over the surface of the mercury. Ten cubic centimeters of mercury in a 100 cc. beaker set under a bell jar with plants caused considerable injury within 48 hours. The same amount of mercury with a centimeter of water over the surface did not injure comparable plants in 30 days.

In a recent publication Ratsek (5) stated that bichloride of mercury was more toxic in light than in darkness and his chemical analyses supported his theory. He mentioned, however, that the air around the plants exposed to bichloride in light was often 18° F. higher in temperature than the air in the dark chamber. The vapor pressure curves illustrated in Figure 4 and the degree of injury at different temperatures illustrated in Figure 2 show the variation that might be expected at 10° difference in temperature. The results of analyses of leaves given in Table V show practically no difference in the amount of mercury taken up by plants in light and dark if the temperature is the same in both cases.

Ratzek (5) suggested, also, that bichloride as such rather than metallic mercury vapor from the reduced compound was mainly responsible for the injury to the plants. The data presented in the present paper show definitely that some reduction occurred, otherwise there would have been no metallic mercury vapor present in the air over soil treated with bichloride. The vapors from the compound itself may play a part, especially at high temperature; at least there is no proof to the contrary. Since, however, bichloride added to tankage is so much more effective than the same amount added to fine sand or powdered charcoal, it is evident that the reduction brought about by organic matter plays the chief part in causing injury to plants. Experimental values for vapor pressures of mercuric chloride below 140° F. are not available. If, however, one may judge from rate of decrease with decreasing temperature indicated by the curves shown in Figure 4, the vapor pressure at room temperature must be very low. According to the International Critical Tables there are no reliable experimental data for vapor pressures of mercuric chloride below 140° F. At this temperature the vapor pressure was found to be 0.00356 mm. compared to 0.02524 mm. for metallic mercury, or it may be stated that the vapor pressure of bichloride is, roughly, only one-tenth that of mercury. From these data it is evident that the possibilities for injury from bichloride at room temperature are very slight.

The vapor pressure of mercurous chloride at room temperature must be extremely small (Fig. 4) and 100 g. or more of the salt were not toxic when sealed under bell jars with plants. If, however, a fraction of a gram of mercurous chloride was mixed with 100 cc. of moist tankage and then enclosed with a plant in a bell jar at room temperature the leaves were severely injured in less than 24 hours and the type of injury resembled that caused by metallic mercury vapor. Therefore, the mercurous chloride must have

been reduced by the tankage to metallic mercury and the vapor therefrom injured the plant. Considering that bichloride is readily reduced by organic matter (4, p. 703) the ultimate results with these two compounds should be the same. This conclusion is further supported by the fact that metallic mercury vapor was detected in air surrounding soil treated with solutions of both mercuric and mercurous chloride.

Tobacco plants exposed to either metallic mercury vapor or vapors from tankage moistened with bichloride solution were injured first on the lowermost leaves. If the treatment continued long enough, say 10 days, the leaves of the middle region on a 12-inch plant showed some injury. The youngest leaves were very resistant and were scarcely, if at all, injured with 14 days of exposure. The question as to what caused the difference arose and analyses of different sets of leaves were made, the results of which are recorded in Table V. There was essentially no difference between the quantity of mercury taken in by the middle and lower leaves, both having absorbed large amounts. The young leaves which were not injured contained less mercury than the other sets but still the amount was approximately four times as much as that found in rose leaves which were badly injured. Tobacco plants removed from the treatment where samples of leaves from other plants had been taken for analyses, given in Table V, grew to maturity. If the mercury remained in the tissues it had no apparent ill effects. Similarly, Jerusalem cherry, mentioned previously as comparatively resistant, continued to flower throughout a four-day exposure though its leaves during that time absorbed from the contaminated air 1315 parts of mercury per million of tissue. Comparable plants receiving the same treatment, but left on an open bench in the greenhouse, showed some ill effects, especially on old leaves, but continued to grow and flower.

It would appear, therefore, that there is no relationship between the susceptible and resistant tissues as to the quantities of mercury which can be tolerated by the different species. The reason for tolerance in one case and susceptibility to injury in the other is not known.

SUMMARY

1. Treating one bed of soil in a rose house with bichloride of mercury injured flowers of Briarcliff variety over the entire range, showing that the plants were attacked by some impurity in the air.

2. Flower buds of roses in all stages of development were affected by the vapors emanating from soil treated with bichloride. Peduncles of very young buds turned yellowish and then black; half mature buds turned brown, and the corollas abscissed from the receptacle without opening; the older buds continued to unfold but the petals lacked the pink color characteristic of Briarcliff variety and brown patches finally developed; stamens also were injured, turning nearly black in half mature buds.

Leaves of rose plants confined in glass cases with treated soil were injured as well as flower buds.

3. Plants of 65 different genera were found susceptible to injury from vapors emanating from soil or tankage moistened with bichloride solution. Ten types were found to be comparatively resistant.

4. The injury to plants from vapors from metallic mercury was similar to that caused by vapors emanating from soil treated with bichloride.

5. Thirteen other compounds of mercury affected plants as reported for bichloride; of these six were organic, and seven inorganic.

6. The extent of the injury and the rate at which metallic mercury injured plants varied with the concentration of the vapor in the air and this in turn varied with the temperature. Vapors from soil treated with mercury compounds injured plants more quickly at high than low temperature. In general, the amount of injury at a given temperature could be correlated with mercury vapor pressure at the same temperature.

7. Metallic mercury vapor was detected in the air surrounding soil treated with the mercury compounds, indicating reduction to the metallic state.

8. Mercury was recovered from leaves of plants confined in glass cases where a small amount of soil had been moistened with a solution of bichloride.

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FORMATION OF CELLULOSE MEMBRANES BY MICROSCOPIC PARTICLES OF UNIFORM SIZE IN LINEAR ARRANGEMENT

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The limiting membrane of the young cotton fiber (*Gossypium hirsutum* L.) is continuous with the lateral walls of the epidermal cell from which it originates and is composed of pectic substance. From the time of the first slight bulging of the outer wall of the epidermal cell, in the direction of fiber elongation, there are, in the cytoplasm, particles of uniform size, separate or in bead-like strands, which are destined to play a most important part in the later development of the fiber wall. The separate particles are ellipsoid in shape and are covered with a thin layer of pectic substance.

In Figure 1 *a* and *e* many such particles are lying approximately parallel to the long axis of the fiber. When this axis is placed at an angle of 45° with a beam of polarized light, the particles are clearly doubly refractive; when the axis is parallel to the beam, they are extinct. Upon the insertion of the selenite plate they show in a faint but definite way the blue color indicated. Their brightness is increased by removal of the pectic material with suitable pectic solvents. Familiarity with their size and general appearance in polarized light renders them readily recognizable in ordinary light.

REFRACTIVE INDEX

The refractive index was measured by the immersion method which has been described fully by Wherry (20). Briefly, the method consists of immersion of the substance in a liquid of known refractive index and observation of the contact line to determine whether the substance has an index higher or lower than that of the liquid. After a few trials a liquid is found in which the substance disappears. The refractive index of the substance then is the same as that of the liquid. Since non-doubly refractive substances have only one index the position makes no difference. But the two or three indices of a doubly refractive substance must be measured at definite positions. The highest and lowest indices are at the positions of extinction in polarized light, with nicols crossed.

A set of liquids with an index difference of 0.005 prepared by A. C. Hawkins (New Brunswick, New Jersey) was used for the measurements.

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The light used was screened through Wratten filter B No. 58 which transmits wave lengths from 490 to 600 $m\mu$ (measured by J. M. Arthur).

Fibrils were separated from the fiber, freed from pectic substance by ammonium hydroxide, washed in water, and dried at room temperature. Several mounts were prepared. If we imagine that the fibril in the upper right quadrant of Figure 1 *c* is being rotated between crossed nicols from 0° to 90° it appears brightest at 45° as shown, but is dark at 0° and 90° . These are the positions of extinction for cellulose (and of other substances having parallel extinction). A fibril is placed in the 0° position at extinction (lengthwise). The upper nicol is slipped out and the refractive index measured. Again with crossed nicols, the fibril is rotated to the 90° position at extinction (crosswise) and the relative values of the indices observed to help in the choice of the first trial liquid. The actual measurement is made on another mount. The refractive indices found for the cellulose fibril are: lengthwise 1.565; crosswise 1.530.

Particles, single and in chains, were squeezed out of the four to six day fiber by pressure. They were then treated with ammonium hydroxide, washed, and dried. The refractive indices were measured on short chains of three to six. The particles are oriented lengthwise in the chain. Its indices seem to be the same as those of a single particle and are much less difficult to determine. The refractive indices of a chain of particles as measured are: lengthwise 1.565; crosswise 1.530. This, in addition to other observations and tests made, leaves no doubt as to the close relation between the particles and the fibril.

The double refraction of the particles, their parallel extinction, and the values obtained for their refractive indices are all indicative of their cellulose nature. Further assurance was sought, however, in microchemical reactions.

SULPHURIC ACID-IODINE TEST

In order to get a clear blue color with this test on the chains of double refracting particles in the young cotton fiber, the usual procedure must be modified somewhat. The proteins of the cytoplasm are precipitated with a yellow to brown color by the I-KI solution. This color and precipitate on the particles together with the blue cellulose reaction gives a greenish color. A short treatment with dilute potassium or sodium hydroxide (10 per cent KOH for 5 min. or 4 per cent NaOH for 10 to 15 min.) prevents the precipitation. Sections of the seed with young fibers are treated on the slide. After washing, a drop of iodine solution is allowed to run under the cover glass, then a drop of the sulphuric acid is added at the edge of the cover glass. In most of the fibers the chains of particles are now a clear cellulose blue, standing out sharply from the almost colorless background of the cell. If the treatment with alkali is too long, the chains of particles are

broken up and the orderly arrangement in the cell is destroyed. When the fibers are a little older (8 to 15 days) there is also a thin layer of cellulose lining the original pectic membrane of the fiber. This thin cellulose membrane swells rapidly and is disrupted in sulphuric acid of the concentration used for cellulose in mature tissue membranes (3 parts acid to 1 or 2 of water). Therefore, the acid is added to the mount in dilute iodine solution, a tiny drop at a time, while watching in the microscope. If the swelling is too slow, another drop of acid is added; if too rapid, water is added. There must be some swelling of the cellulose or there is no blue color with iodine but too much swelling destroys all structural features.

PECTIC SUBSTANCE

Pectic substance was identified by its non-double refraction and by its solubility in dilute solutions of potassium hydroxide, sodium hydroxide, and ammonium hydroxide. Ruthenium red was used for localization of the substance before treatment with alkali and for demonstration of its absence after treatment. Although no chemical analyses of the galacturonic acid content were made, the solubility, non-double refraction, and staining with ruthenium red are properties like those of the pectic substance in apple fruits and other tissues which have been analyzed (4, 13). Therefore we believe that the substance covering the cellulose particles, the cellulose fibrils, and the fiber itself is pectic substance. It is not the water soluble pectin, but whether it is pectic acid, a pectate, or protopectin is not known. When a nearly mature fiber from an unopened boll is put into a 4 per cent solution of sodium hydroxide, the separation of the fiber into fibrils and of the fibril into particles does not occur simultaneously. First there is the separation of the fiber into fibrils by partial solution of the pectic substance surrounding them. Then, an hour or more later, the fibril begins to separate into particles. A dry fiber from an opened boll requires much longer treatment to bring about the same separation.

FIBRIL STRUCTURE

Optical and microchemical reactions serve to establish the cellulose nature of the particles which are found in the cytoplasm of young fibers. The possibility of a relationship between these more or less free cellulose particles and the compact cellulose membrane of the mature fiber wall immediately suggests itself. In order to establish such a relationship the microchemical and optical properties of the particles and of the mature wall must be compared.

When the fiber is five or six days old the limiting membrane is no longer entirely pectic. Closely affixed to its inner side is a thin layer of fibrils which react positively to the previously mentioned optical and microchemical tests for cellulose. The non-doubly refractive pectic por-

tion of the wall and the doubly refractive cellulose layer are clearly differentiated in polarized light. In the plasmolyzed state, the protoplast draws away from the thin cellulose lining, thus showing unmistakably the dual nature of the young membrane.

It is in the turgid cell, however, that one sees the relationship between the particles and this newly formed lining to the pectic wall. Figure 1 *a*, an optical section slightly below the surface of the protoplast, shows the particles in the various states in which they have been observed in the young cell. Toward the center they are separate and in short chains. In the extreme outer regions of the protoplast the chains are longer, and in some instances, near to the wall the particles have entered into the formation of fibrils. During plasmolysis these chains and newly formed fibrils are often drawn away from the wall with the protoplast; in others they adhere to the wall; while not infrequently they are "stranded" between the two (Fig. 1 *e*).

From direct observation we may conclude, therefore, that the pectic-coated cellulose particles distinguishable in the living cytoplasm may exist singly or in chains; that toward the center of the lumen the single and short chain types of arrangement predominate; that as the wall is approached the chains become longer; and that a single chain of closely appressed particles forms a single fibril of the fiber wall. The shape of the separate particle is more nearly ellipsoid than spherical and an arrangement in which the long axis of the ellipsoid is parallel to the long axis of the chain seems to be the typical one. These observations are presented at this time without reference either to the origin of the particles or to the mechanism by means of which the different types of arrangement may take place.

It is obvious that a membrane so constructed should lend itself to separation in such a way that both the fibrils and the particles might be reidentified. This is accomplished by either of two methods, the one used depending upon the material. If mature fibers are taken from an unopened boll before the process of dehydration has progressed to an appreciable extent, the separations are accomplished without the use of pectic solvents. If mature dried fibers are used, mild pectic solvents must be employed to soften the cementing materials. Figure 1 *f*, *h*, and *o* represent moist fibers from an unopened boll. If one end of such a fiber is held between the thumb and forefinger, and is then broken over the edge of the thumb nail by pulling upon the opposite end with fine tweezers, the break in the outer pectic coating of the fiber and the breaks in the individual fibrils will rarely coincide. In Figure 1 *f* the pectic layer forming the original limiting membrane as well as the pectic covering of the protruding fibrils are made conspicuous by staining with ruthenium red. Figure 1 *h* shows the same staining property of the single particles. In Figure 1 *o*, the unstained fibrils

are loosened and slightly separated. At various points the disintegration of fibrils into particles is observed. These disintegrations may be brought about by slight pressure upon the cover glass of the mount.

Separation by means of pectic solvents is illustrated in Figure 1 *b*, *c*, *d*, *k*, and *m*. The first marked change consists in a straightening of the fibrils from the typical spiral position to the one parallel to the fiber axis shown in Figure 1 *d*. Two small strands of such fibrils placed at right angles to each other and, in turn, at 45° to the beam of polarized light are illustrated in Figure 1 *b*. Complementary colors are seen in the presence of the selenite plate. A single fibril, photographed in the 45° position in each of the four quadrants, further illustrates the optical properties of the fibril (Fig. 1 *c*). The smooth surface and the coloration in the different positions are indicative of the uniformity in size of its constituent particles and the regularity with which they are arranged. Pressure upon the cover glass causes some of the particles to separate. Single particles frequently float away from the fibril mass thus affording excellent conditions for individual observation. Such a particle is in clear focus in the first quadrant of Figure 1 *k*. The similarity in optical behavior of the particle, fibril, and more or less intact fiber mass may be seen at a glance. The same field photographed after the removal of the selenite screen (Fig. 1 *m*) reveals only those areas in which the particle and fibril arrangement approached the position of greatest brightness. The reaction of the particles to the sulphuric acid-iodine reagent furnishes additional evidence of their cellulose nature. This is illustrated in Figure 1 *n*. No way was found to measure directly a single particle. The length of many chains of three and six was measured, however, by means of an ocular micrometer. From these values the length of a single particle was calculated to be approximately 1.5 microns.

In Figure 1 *g*, a paraffin section of a 4-day cotton ovule is used to illustrate the part played by the cellulose particles in building up the membranes of other tissues in the cotton seed. In the upper portion of the field are masses of young cotton fibers showing numerous long chains of cellulose particles. Below these are the epidermal cells in whose walls and cytoplasm cellulose particles may also be distinguished. In the lower part of the field a similar condition is seen to obtain in the parenchyma cells.

The observations and illustrations presented thus far serve to indicate the rôle played by the cellulose particle in building the limiting membranes of the cotton fiber. Observations of cellulose membranes from cells in many other parts of the plant kingdom have revealed in every instance the presence of particles of the same size and shape with similar optical and microchemical properties. A few examples will serve to make more specific these assertions.

ASPERGILLUS

At the verbal suggestion of Doctor Charles Thom² the sporangiophores of several species of *Aspergillus* have been examined for "spiral markings in the walls similar to those in the cotton fiber." Cellulose membranes have been found in the sporangiophores of *A. niger*, *A. ochraceous*, and *A. flavus*, but not in the hyphae, except for a distance of 10 to 20 microns on either side of the point of origin of the sporangiophore.

The very young sporangiophore presents an appearance similar to that of the young cotton fiber; a thick wall of pectic substance and numerous cellulose particles in the cytoplasm, singly, and in chains. The lining of the pectic membrane with cellulose seems, however, to follow a different procedure than in the cotton fiber in which the cellulose appears simultaneously over the entire inner surface of the membrane. This contrasting behavior in *Aspergillus* is illustrated in Figure 2 *h*, *k*, and *o*. The cellulose appears first in the basal portion of the young sporangiophore. Its presence and relative thickness are indicated by the appearance in polarized light (Fig. 2 *h*). In the tip of the same sporangiophore the cellulose lining of the pectic membrane has not yet appeared. The gradual appearance of the cellulose nearer to and finally in the membrane of the developing columella, as well as the increase in thickness of the cellulose portion of the membrane as sporangiophore development continues, are illustrated in Figure 2 *k* and *o*.

Cellulose particles may be seen in both portions of the young sporangiophore (Fig. 2 *h*), and in the basal portion of *k*. Because of its doubly refractive glow the outline of the mature columella may be detected even through the dense mass of spores in Figure 2 *o*. In Figure 2 *f* (stained with ruthenium red) we see in optical section, the outer membrane of pectic substance, the thickening cellulose lining, and, toward the center, cellulose particles with their stained individual coats. In Figure 2 *e* there are separate particles and strands of particles near the tip. Below these, toward the base of the stalk we see the region of transformation of strands of particles into fibrils of the mature wall. The same type of gradient at higher magnification is shown in a portion of another sporangiophore, Figure 2 *g*.

The striking similarity between the arrangement of fibrils in the membrane of the sporangiophore of *Aspergillus* and in the cotton fiber is illustrated in Figure 2 *m* and *n*. The crossed spirals, the slope of the spirals, the areas of reversal, so typical of a cotton fiber (6), are all represented here.

HIBISCUS

In the hairs formed upon the surface of the seed of *Hibiscus*, so closely related to cotton, we might expect to find a similar type of spiral architec-

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ture in the formation of the cellulose membrane. This is true, with one marked exception. There are no areas of reversal in the direction of the spirals. Figure 2 *s* illustrates both the similarities and the difference. The spiral structure is readily brought into evidence by slight pressure upon the cover glass of the mount. The regularity and compactness with which this wall is built can be observed through low magnification of a portion of an intact hair in which the degrees of thickness of the wall are represented by the different areas of polarization colors extending parallel to the long axis of the cell on either side of the lumen (Fig. 2 *r*).

Figure 2 *p* is another portion of a *Hibiscus* hair, stained with ruthenium red, illustrating the comparatively intact condition of the outer pectic covering of the cell. The condition of this pectic material is of particular interest because of the age of the specimen. Along with samples of many other forms closely related to cotton, this specimen of *Hibiscus spathecus* has been provided through the courtesy of Doctor E. D. Merrill of the New York Botanical Garden. It was taken from a herbarium specimen collected in 1830.

By the use of mild pectic solvents the walls of these hairs of *Hibiscus* were separated into cellulose fibrils and the fibrils, in turn, into cellulose particles, exhibiting no differences in either their appearance or their optical and microchemical reactions from those from the cellulose walls of the cotton fiber.

ORIENTATION IN OTHER CELLULOSE MEMBRANES

The thin cell membranes of *Spirogyra* are doubly refractive, exhibiting parallel extinction and having the greatest brightness at 45° to the plane of polarization. The middle lamella of non-doubly refractive material appears as a thin dark line between the cellulose end walls of two adjacent cells. This middle lamella as well as the outer sheath of pectic material stains with ruthenium red. The reaction of the cellulose portion of these membranes to the sulphuric acid-iodine test is typical. A clear cellulose blue was produced in every part of the swollen membrane. The cellulose particles are most readily observed in young cross walls since the spiral plastids in the periphery of the cytoplasm tend to obscure vision along the lateral walls. The chains of particles making up these cross walls show a coloration complementary to that of the lateral walls in the presence of the selenite plate. This illustrates the tendency of the particles in such cylindrical cells, to maintain a position in which their long axes are parallel to the surface of the protoplast. A similar tendency may be observed in multicellular tissues whose cells are more irregular in shape. All portions of the walls parallel to these cross hairs are extinct in the absence of the selenite plate, while the remaining portions of the wall, in the presence of

the selenite plate, are either blue or yellow, depending upon their position relative to the beam.

The cell wall of *Valonia ventricosa* lends itself most readily to dissection. A portion of dried material, moistened thoroughly with warm distilled water, may be separated with fine needles into its constituent fibrils. The addition of a trace of KOH to the distilled water is helpful. The optical and microchemical properties of the fibrils indicate that they are of cellulose with a thin coating of pectic material. Slight pressure upon the cover glass brings about the separation of some of the fibrils into their constituent particles. That these individual particles, in turn, are composed of cellulose with a pectic coating is shown by optical methods in both ordinary and polarized light and by microchemical tests. An illustrated discussion of cellulose wall formation in *Spirogyra*, *Oedogonium*, and *Valonia*, is to be published separately.

Among the many other cells with cellulose membranes which have been examined in connection with this study are the root hairs of corn, tomato, and tobacco, and the epidermal hairs of cucumber, cotton, and tobacco. In every instance the membranes have been found to consist of cellulose particles in linear arrangement. Examination of individual particles has revealed the presence of a non-doubly refractive coating upon each one. This coating has reacted positively to the microchemical tests for pectic substance which have been described.

CELLULOSE-FORMING BACTERIA

It has been of interest in this connection to examine the bacterial cellulose membranes in which the cellulose formation has been considered to be intercellular, having been synthesized directly from the sugar molecules in the nutrient medium and deposited in the medium in long, well oriented chains of cellulose unit cells (7).

The material has been supplied by Doctor H. L. Hibbert³ from his cultures of *Acetobacter xylinus*. The results, briefly, are as follows: The entire "membrane" has been found to consist of bacterial organisms with no true intercellular substance. The single bacterium is composed of a protoplast surrounded by a cellulose membrane which in turn is covered with a layer of pectic substance. This pectic coating is not thick enough to obscure the double refraction of the cellulose membrane in polarized light. The individual organisms shown in Figure 2 *a* and *b* illustrate this optical behavior. In the first quadrant of Figure 2 *a*, a single bacterium whose long axis is at 45° to the beam of polarized light is blue. Several other bacteria whose long axes approach the perpendicular to the axis of this one are distinctly yellow. Obviously they are not in the position of greatest brightness. In

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the second quadrant is a bacterium whose long axis is parallel to the direction of the beam. With the selenite plate its color is that of the background; when the selenite plate is removed, only those in the first quadrant are visible (Fig. 2 *b*), and they in a degree of brightness consistent with their orientation with respect to the direction of the beam. Figure 2 *c* illustrates the sulphuric acid-iodine reaction of the cellulose wall of the bacterium. Small portions of chains, stained with ruthenium red, are shown in Figure 2 *d*. This staining property as well as solubility tests were used to determine the presence of the pectic substance.

At low magnifications the individual bacteria cannot be seen and consequently the position of the cellulose cannot be determined. Observations of thin portions of intact membranes under such conditions do indicate, however, the extreme regularity in directional arrangement of the strands of bacteria by the uniformity of coloration over the entire field.

DISCUSSION

The cellulose particles which we are describing here are not the regularly arranged structures of molecular dimensions which diffract X-rays. They are presumably aggregates of these cellulose unit cells which play an important rôle in both their sub-microscopic and microscopic orientation. Regardless of the ultimate structure of the particles, however, they apparently represent standard units which are used in building up the more or less intricate structure which we recognize as a cellulose membrane. The different lines which are followed in this form of architecture throughout the plant kingdom are varied and can be determined most accurately by further observational research.

The fundamental contributions to cellulose analysis which have been made through the technique of X-ray diffraction are in no sense controverted by the presence of the cellulose particles in the membrane. It is obvious, however, that many of the theoretical phases of this branch of cellulose research are rendered untenable in the light of this new conception of fibril structure. Since the observation of particles in many cellulose membranes indicates an adherence to a linear arrangement, and since their appearance in polarized light shows a constancy in the position of the long axis of the particle with respect to the long axis of the chain, we may infer that the cellulose particles play an important part in maintaining the orderliness of sub-microscopic unit cell arrangement so evident in diffraction analyses of cellulose membranes. It is clear, moreover, that interpretations of sub-microscopic data do not become independent of the fibril through the knowledge that it may be separated into cellulose particles of uniform size and shape. This new information concerning fibril structure should be helpful in the interpretation of any diffraction patterns which have been obtained from known material under carefully controlled con-

ditions. The possibility of experimental manipulation of the particles should be even more useful in future research in these fields.

These optical and microchemical studies of cellulose particles have been attended by a continuous effort to obtain additional information which would have some bearing upon the currently accepted "micellar" hypothesis. The presentation of conclusions in this connection may be preceded by a few quotations from a standard textbook of mineralogy (5):

(p. 7) "A crystal is the regular polyhedral form, bounded by smooth surfaces, which is assumed by a chemical compound under the action of its intermolecular forces, when passing, under suitable conditions, from the state of a liquid or gas to that of a solid.

"... a crystal is characterized first by its definite internal molecular structure, and second, by its external form.

(p. 8) "When a mineral shows no external crystalline form it is said to be massive. It may, however, have a definite molecular structure and then it is said to be crystalline. If this structure as shown by the cleavage or by optical means, is the same in all parallel directions through the mass, it is described as a single individual. If it varies from grain to grain, or fiber to fiber, it is said to be a *crystalline aggregate*, since it is in fact made up of a multitude of individuals."

Cellulose, by reason of both X-ray and optical determinations, would seem to fulfill in the strictest sense the necessary qualifications of a crystalline substance. The various types of cellulose membranes made up as they are of individual crystalline particles would, in this sense, represent crystalline aggregates.

DEVELOPMENT OF IDEAS REGARDING ORIGIN OF CELLULOSE

Ideas with respect to the structure and mode of formation of cellulose membranes began to take definite form about one hundred years ago. Valentin (19), a biologist, recorded a noteworthy observation in 1837. From microscopic studies of cell membranes in ordinary light he concluded that granules which he saw in the cytoplasm build cross lines which later become the spiral lines of the cell membrane. Agardh (1) demonstrated in 1852 the fibrillar nature of the membrane material through the separation of these spiral "lines." Von Mohl (8, 9) whose writings touch upon all phases of cellular phenomena of current interest in that period, corroborated the findings of Agardh, but disagreed with the observations of Valentin. Pringsheim (14) stated in 1854 that the cell wall layers are formed from the outer portion of the protoplasm.

In order to interpret the optical and swelling phenomena in both cellulose and starch Nägeli (10, 11) considered it necessary to postulate the existence of sub-microscopic crystalline "micellae." This micellar hypoth-

esis has not been acceptable to many of the botanical investigators of cell membrane structure. Among the more prominent of these are Strasburger (18), who disagreed with Nägeli and in 1882 announced the observation of protein "microsomes" in the outer portion of the cytoplasm which he believed played an important part in wall thickening, and Wiesner (21) who expressed the opinion in 1886 that the protein microsomes or "dermatoplasma" change to cellulose "dermatosomes" after they have become a part of the membrane. Both the microsomes in the cytoplasm and the dermatosomes in disintegrated mature walls were microscopically visible and were suggested from direct observation in contrast to the theoretical sub-microscopic crystalline micellae.

Ambronn (2, 3), over a period of forty years of intensive optical research upon cellulose and many other substances, has contributed much to our information concerning the arrangement of doubly refractive material in the cellulose membrane, the uniform optical behavior of the fibril, etc. Unable to observe microscopically any units smaller than the fibril which were playing a part in the crystalline orientations concerned, he has developed, in connection with his interpretations, not the conception of visible microsomes or dermatosomes, but that of the invisible micellae of Nägeli. To Ambronn and his coworkers we may largely attribute the revival of the micellar hypothesis between 1890 and 1913. In the latter year Nishikawa and Ono (12) published their results in connection with the X-ray diffraction analysis of cellulose fibers. The symmetrical patterns obtained contributed new and important evidence as to the crystallinity of cellulose. Since that time a very large number of papers has appeared from chemical and X-ray laboratories in many parts of the world in which the authors have attempted to correlate their diffraction data and various physical properties of cellulose with the crystalline micellae of Nägeli and the micellar orientations of Ambronn. Notable among X-ray investigators who have not subscribed to the micellar hypothesis is Sponsler to whom we are indebted for the first approximate dimensions of the cellulose unit cell (15, 16). He believes that the diffraction data obtained from X-ray analysis of cellulose may be accounted for upon the basis of chains of cellulose unit cells of indefinite length, thus eliminating the necessity for consideration of micellar aggregates. According to his recent study of the growth of cross walls in certain algae (17), the deposition of cellulose takes place in the form of molecular layers at the rate of 20 molecular layers per second.

This brief review of previous work will serve to place the cellulose particle in its proper relationship to both past and present considerations in the field of cellulose analysis. An outline form (Table I) shows concisely the principal contributions. It is very probable that Valentin saw and correctly interpreted the function of the "granules" almost one hundred years ago; that the "microsomes" were the cellulose particles whose chemi-

TABLE I
DEVELOPMENT OF IDEAS REGARDING ORIGIN OF CELLULOSE

Author	Date	Material	Methods	Conclusions
Valentin	1837	Cell walls	Microscopic	Granules in cytoplasm build cross lines which later become spiral lines of cell membrane
Agardh	1852	<i>Conserva melagonium</i>	Microscopic	Cell walls separable into spiral fibrils
Nägeli	1858 1863	Starch grains Cell walls	Microscopic swelling phenomena	Sub-microscopic crystalline micellae to explain double refraction and swelling phenomena
Strasburger	1882	Cell walls	Microscopic	Microscopic protein "microsomes" in "Hautschicht" play important rôle in wall thickening
Wiesner	1886	Cell walls Fibrils	Microscopic	Cell walls composed of microscopic cellulose "dermatosomes"
Ambronn	1892 1925	Cell walls Fibers	Microscopic	Cellulose composed of sub-microscopic anisotropic crystalline micellae in orderly arrangement within the fibril
Nishikawa and Ono	1913	Wood fibers Bamboo fibers	X-rays	Symmetrical diffraction pattern contributes additional evidence to crystallinity of cellulose
Sponsler	1925 1926	Ramie	X-rays	Approximate dimensions of elementary cell of cellulose $6.10 \times 5.40 \times 10.25 \text{ \AA}$

cal make-up was incorrectly considered to be protein; that Wiesner failed to recognize the cellulose nature of the "dermatoplasma," and that the emphasis placed upon the micellar hypothesis has served to obscure the importance of the observational data which these investigators contributed. After an interval of almost fifty years during which microscopic structures smaller than the fibril have received little attention in the field of cellulose analysis, these structures which enter into the formation of the cellulose membrane are now demonstrated in the form of *visible anisotropic crystalline particles* of uniform size in linear arrangement in both the living cytoplasm and in their final position in the mature cell wall.

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EXPLANATION: FIGURE 1

a. Tip of young cotton fiber showing cellulose particles separate, in chains, and in the process of fibril formation. Polarized light with selenite screen. $\times 1020$.

b. Fibrils from mature dried cotton fibers cut, ground, and treated with ammonia containing 20 per cent ammonium thiocyanate (6). Bundles at right angles in polarized light. $\times 1150$.

c. Single fibril from *b* showing optical behavior in four positions of greatest brightness in polarized light. $\times 1080$.

d. Cotton fiber cut, ground, and treated with ammonium thiocyanate in ammonia (see *b*) showing change from spiral to parallel position of fibrils. $\times 1350$.

e. Portion of cotton fiber from 11-day boll showing membrane formation by cellulose particles. $\times 1495$.

f. Broken fiber from 40-day cotton boll before dehiscence stained with ruthenium red to indicate original pectic membrane of the fiber and the pectic coating upon the separate fibrils. $\times 1495$.

g. Section of 4-day cotton ovule from paraffin material showing cellulose particles in young fibers, epidermal cells and parenchyma cells. $\times 1265$.

h. Particles and fibrils from mature cotton fiber taken from a mature boll before dehiscence treated for 6 hours with dilute ammonium hydroxide, and stained with ruthenium red. $\times 1380$.

k. Single particle, chains of particles and fibrils from mature cotton fiber treated similarly to *b* and slightly crushed with the coverglass. Polarized light with selenite screen. $\times 1000$.

m. Specimen as in *k* without selenite screen. $\times 1000$.

n. Particles in fiber from 11-day cotton boll showing sulphuric acid-iodine reaction. $\times 1495$.

o. Separation of mature cotton fiber into fibrils and particles by treatment as in *b*. Ordinary light.

EXPLANATION: FIGURE 2

- a. Single bacteria from cultures of *Acetobacter xylinus* in positions of extinction and brightness in polarized light with selenite screen. $\times 2700$.
- b. Mount shown in a without selenite screen. $\times 2700$.
- c. Sulphuric acid-iodine reaction in *A. xylinus*. $\times 1950$.
- d. Pectic coating upon the surface of *A. xylinus* stained with ruthenium red. $\times 1950$.
- e. Cellulose particles separate and in chains in young sporangiophore of *Aspergillus niger*. Polarized light. $\times 700$.
- f. Portion of young sporangiophore of *A. niger* showing original pectic membrane and pectic coating upon the individual cellulose particles stained with ruthenium red. $\times 1150$.
- g. Cellulose particles in the process of fibril formation in sporangiophore of *A. niger*. $\times 1900$.
- h. Tip and base of young sporangiophore of *A. niger* showing earliest cellulose membrane formation near base of stalk. Polarized light. $\times 1700$.
- k. Developing sporangiophore of *A. niger* showing increasing thickness of cellulose membrane in lower portion. Polarized light. $\times 1700$.
- m. Portion of stalk of mature sporangiophore of *A. niger* showing crossed spiral arrangement and reversal area of cellulose fibrils. Polarized light. $\times 1150$.
- n. Another portion of a mature sporangiophore of *A. niger* showing more frequent areas of reversal. Polarized light. $\times 1150$.
- o. Mature sporangiophore of *A. niger* showing thick cellulose membrane throughout its entire length. Polarized light. $\times 500$.
- p. Portion of fiber of *Hibiscus spathecus* stained with ruthenium red to indicate its outer pectic layer. $\times 700$.
- r. Portion of intact fiber of *H. spathecus* showing longitudinal areas of coloration in polarized light. $\times 200$.
- s. Base of *H. spathecus* fiber slightly crushed to bring out crossed spiral arrangement of fibrils. $\times 550$.

DWARF SEEDLINGS FROM NON-AFTER-RIPENED EMBRYOS OF PEACH, APPLE, AND HAWTHORN

FLORENCE FLEMION

Many seeds require special treatment before they will germinate. This may be due to an inhibiting effect of the seed coat, or to a dormant or an undeveloped embryo which requires a period at low temperature before germination takes place, or to a combination of these factors (1). In seeds of *Rhodotypos kerrioides* Sieb. & Zucc. both the seed coat and the embryo play a rôle in the dormancy (2). It has been possible, however, to produce seedlings from freshly-harvested non-after-ripened seeds (3) by removing the seed coats and placing the embryos in a moist medium at 25° C. The seedlings obtained in this way were different from seedlings from after-ripened seeds in that they had short stocky internodes and a stunted appearance characteristic of dwarfs.

The plants obtained during the fall and winter began to grow normally in the spring. Seedlings produced in the spring from non-after-ripened seeds (the seeds had meanwhile been stored air dry in open containers at room temperature) did not show such extreme stunting. These results indicate a possible effect of length of day or quality of light, although it must not be overlooked that the normal growth in the seedlings planted in the spring may be a result of changes which have taken place in the seed during the interval from the time of harvest. Preliminary experiments (unpublished) in which dwarf seedlings have been exposed to a longer day during the winter and in which seedlings obtained from after-ripened seeds have been exposed to very short days have shown that light is not the determining factor.

It is important to note that in this case as well as in those given below, there results finally a normally growing seedling although neither the seeds nor the young seedlings have been subjected to low temperature.

The experiments reported in this paper show that seedlings obtained from freshly-harvested non-after-ripened seeds of the peach (*Prunus persica* [L.] Stokes), apple (*Pyrus malus* L. variety unknown), and hawthorn (*Crataegus* L. variety probably *punctata*) are also dwarf-like in character. The conditions necessary to obtain plants are given, together with photographs of typical examples.

For breaking the hard outer coats (pericarp) of peach and hawthorn, a machine was constructed with a lever which operates an eccentric shaft so that a plunger breaks the coat at the lower point of eccentricity and is thereby mechanically prevented from crushing the embryo.

RESULTS

With peach seeds. Seedlings from non-after-ripened peach seeds, which normally require two to three months at 5° C. for germination to take place, can be obtained as follows: The hard outer coat (pericarp) is removed, the embryo is soaked overnight in tap water after which the inner coat is peeled off, and the naked embryo placed in moist peat moss at 25° C. It is important that the peat moss contain ample moisture but it should not be too wet; otherwise a considerable number of the embryos will be killed by fungi. As germination takes place (80 to 90 per cent in five to seven days) the seedlings are transferred to soil and placed in the greenhouse.

In this way hundreds of seedlings have been produced from freshly-harvested seeds of the 1932 and 1933 crops. The varieties used included Belle of Georgia, Early Crawford, Elberta, and a number of unknown varieties. These plants exhibited abnormal stunted growth until spring when normal growth began. Whenever possible (when experiments were started two or more months after harvest), seedlings from after-ripened seeds were placed side by side with the dwarf seedlings. The difference in the rate of growth was so marked that all of the dwarf seedlings were much smaller than the smallest of the seedlings from the after-ripened seeds.

Three typical plants from non-after-ripened seeds are shown in the upper part of Figure 1. As seen from left to right the plants were planted in October, January, and May respectively and the photographs (one-quarter natural size) were taken when the plants were four months old. The lesser tendency of seeds planted in the spring to produce dwarfish plants is illustrated by the better growth of the plant on the right as compared with the other plants of the same age but planted in the fall and winter. A four months old seedling from an after-ripened seed (three months at 5° C. in moist peat moss) is shown on the lower right in Figure 1. This was planted in January at the same time as the seedling from non-after-ripened seed shown in the upper center. The photograph in the lower left illustrates the type of growth taking place in the dwarf plants in the spring. The plant illustrated is the same plant as in the upper left, but three months later. The growth now appears normal in every way.

With apple seeds. In a similar manner dwarf-like seedlings were produced from apple embryos although the number of seedlings obtained was less than in the peach since only about 20 per cent of the embryos germinated without after-ripening. The upper row in Figure 2 (from left to right) shows (one-half natural size) a dwarf-like seedling eight months old from a non-after-ripened seed and a normal seedling five and one-half months old from an after-ripened seed (two and one-half months at 5° C. in moist peat).

With hawthorn seeds. When hawthorn seeds of the 1932 crop, with outer coat (pericarp) and inner seed coat removed, were placed at 25° C. in moist peat moss, no development occurred. If the embryos without the inner coats were placed at room temperature in tap water through which

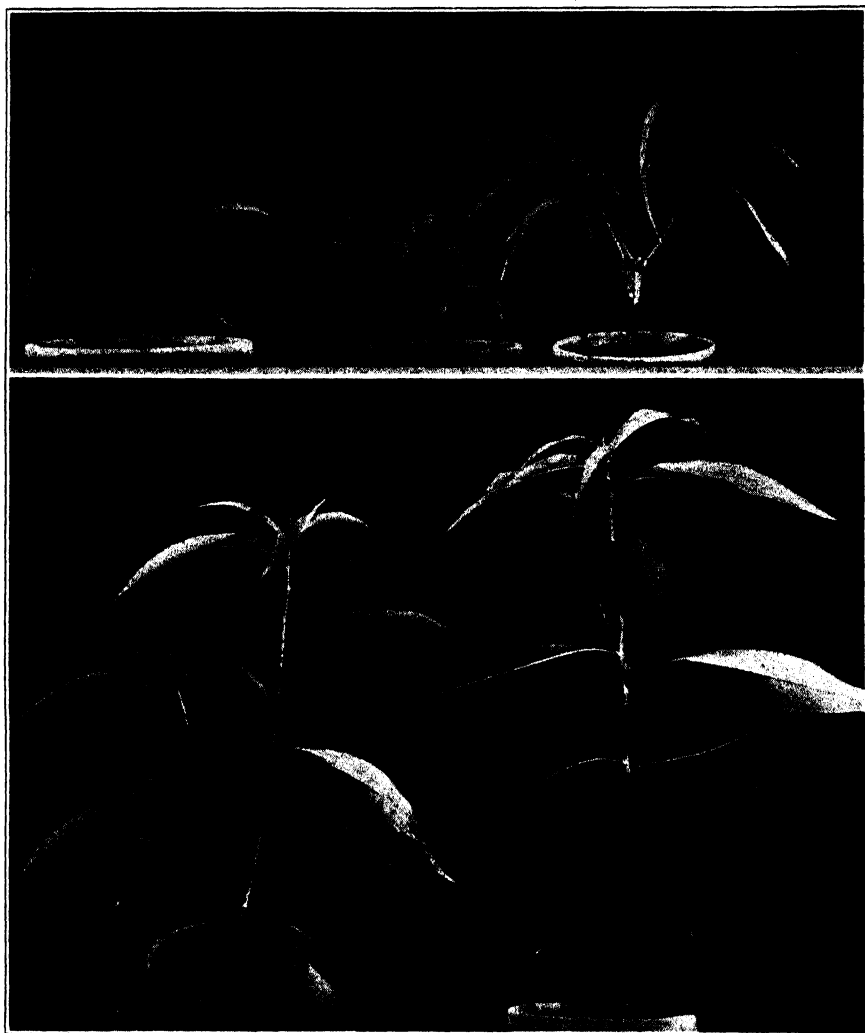


FIGURE 1. Dwarf-like seedlings (one-quarter natural size) from non-after-ripened seeds of peach. Upper half, four months old seedlings started (reading from left to right) October, January, and May, respectively. Lower left, same as upper left but three months later. Lower right, four months old seedling from after-ripened seed placed in greenhouse at same time as non-after-ripened seed resulting in plant (at four months) shown in upper center.

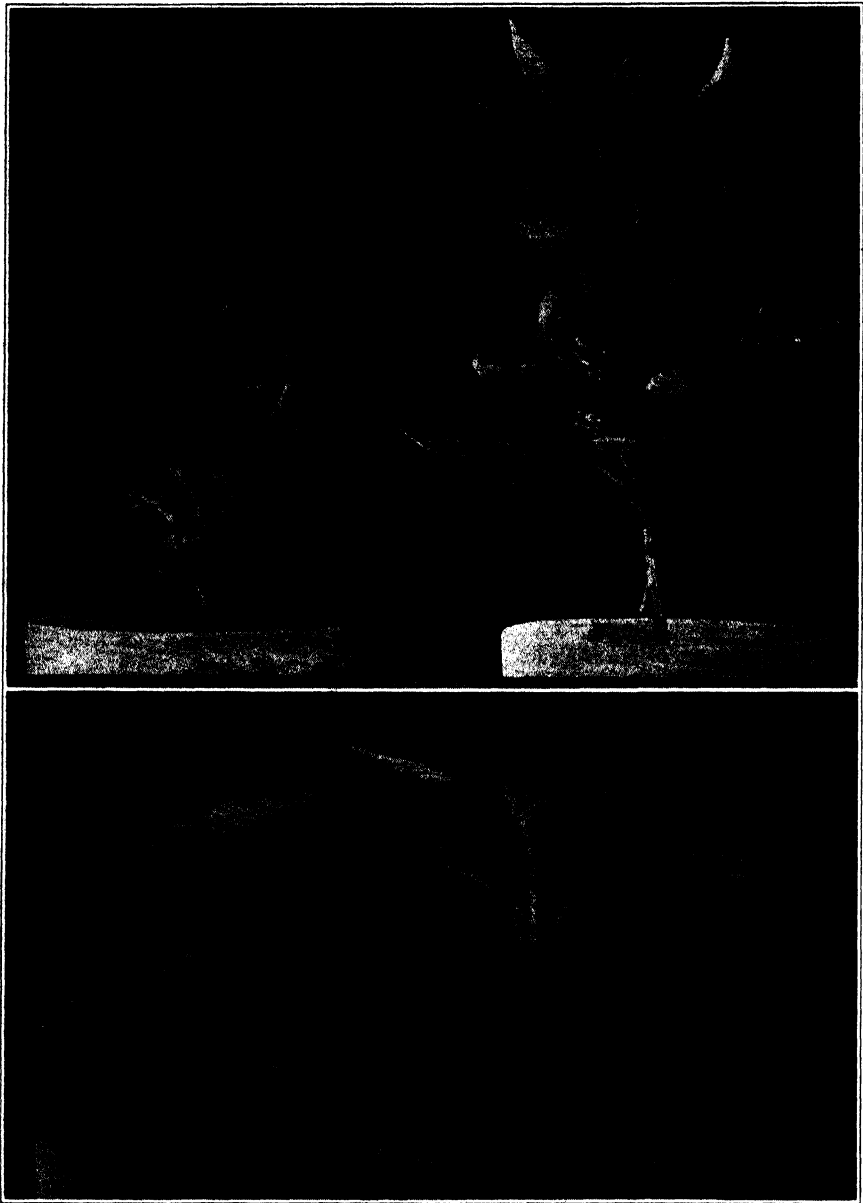


FIGURE 2. Upper row, apple seedlings. Left, an eight months old seedling from non-after-ripened seed and right, a five and one-half months old seedling from an after-ripened seed (one-half natural size). Lower row, hawthorn seedling from a non-after-ripened seed photographed when six and nine months old (one-half natural size).

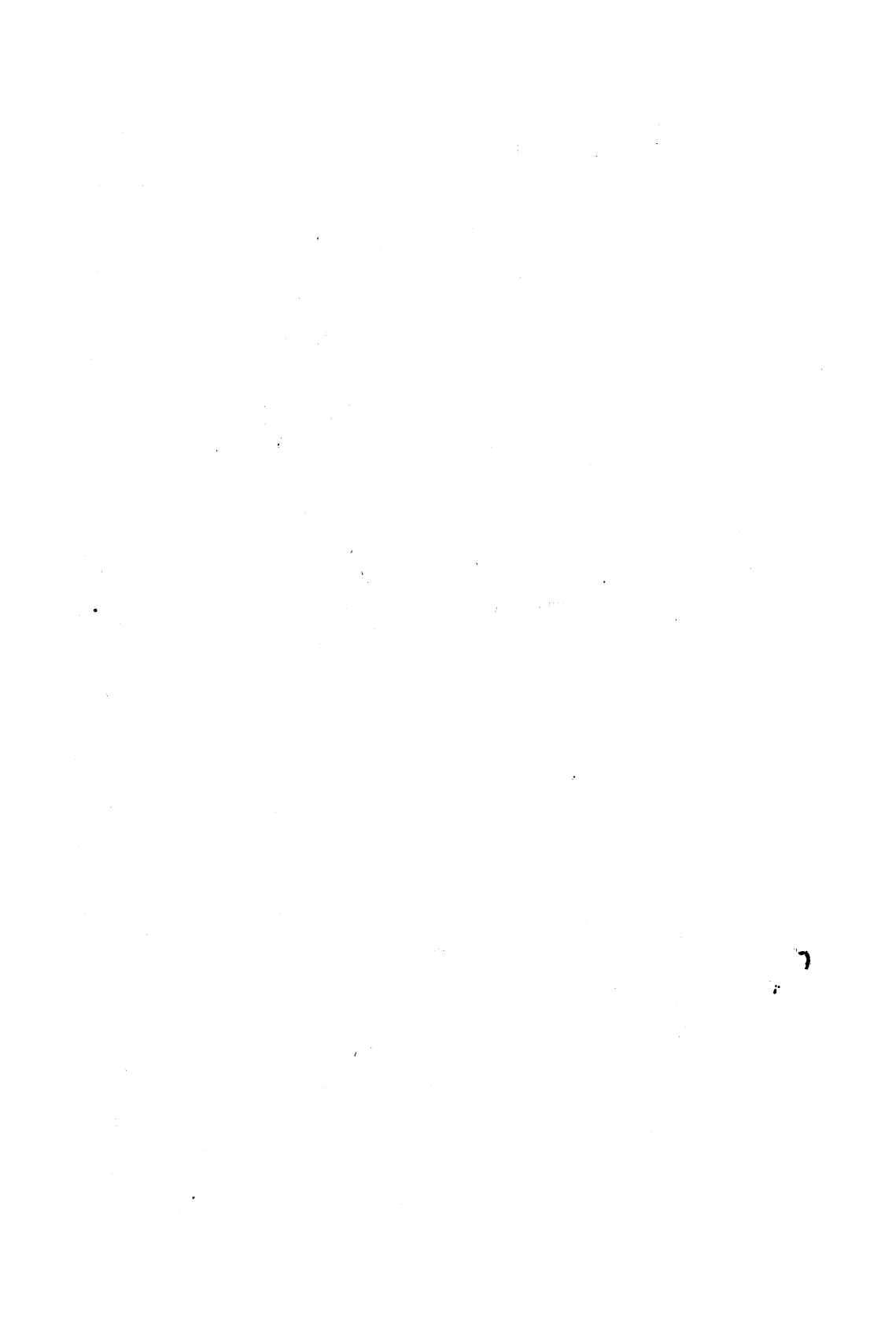
air was continuously passed for four or five days and then transferred to moist filter paper in petri dishes, the hypocotyls developed in about ten days. The lower row in Figure 2 is a photograph of one such plant (one-half natural size) obtained in this way and photographed when six months old (March 14) and again at nine months (June 16). The assumption of normal growth by dwarfish seedlings in the spring is thus also observed in hawthorn.

SUMMARY

Seedlings can be obtained from freshly-harvested seeds of peach, apple, and hawthorn, which normally require an after-ripening period of several months at low temperature, by removing the outer hard coat and inner seed coat and placing the embryos under germinative conditions. Such seedlings exhibit a very abnormal growth which may be described as dwarf-like in character. After some months, however, apparent normal growth begins.

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HISTOPATHOLOGY OF INSECT NERVE LESIONS CAUSED BY INSECTICIDES¹

ALBERT HARTZELL

The writer has previously demonstrated (21) the presence of nerve lesions in the ventral ganglia of meal worm larvae (*Tenebrio molitor* L.) killed by pyrethrum concentrates. It seemed desirable to pursue this investigation further and determine more precisely the regions of the nervous system affected. It would be of special interest to ascertain, for example, whether the destruction of nerve tissue is localized in certain ganglia since this knowledge might indicate the manner in which the pyrethrins cause death to insects. Although it is shown by Krüger (10) that these lesions are not necessarily restricted to nerve tissue, attention in this study was confined to their location in the central nervous system as it was thought that destruction of this tissue would prove more likely to be a primary factor in bringing about death than lesions in other tissues.

Barlow (2) divided the abdomen of a dragon fly and found that decapitation did not stop respiratory movements, but only lessened their force, indicating that the seat of control is in the thorax and abdomen and not in the head. This was later confirmed by Plateau (13) who conducted an exhaustive study on the respiratory movements in insects which has hitherto not received the recognition that it deserves. By means of destroying individual ganglia in living insects this writer was able to demonstrate that each ganglion of the ventral nerve cord is the respiratory control center for its respective segment. His experiments indicate that the seat of respiratory control is located primarily in the central nervous system and not in the sympathetic system as was previously supposed.

As no microchemical test of sufficient accuracy is known that will detect the presence of the pyrethrins in insect tissue, the present investigation deals with a histological rather than a cytological approach of this problem. Attention has been, therefore, directed on the detection of lesions rather than a complete investigation of the cellular structure of the tissue. No study of sublethal doses was made. The effects of rotenone, triorthocresyl phosphate, high radio frequency, and heat were compared also with that produced by pyrethrum concentrates on nerve tissue. In addition, comparison was made of the action of pyrethrum concentrates on the contraction of frog's gastrocnemius muscle and a veratrine-treated muscle with that of a normal muscle.

¹ Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 73.

MATERIAL AND METHODS

In general the same technique was used in this work as has been described previously (21). Emphasis was placed on detecting lesions throughout the main regions of the central nervous system of adult red-legged grasshoppers (*Melanoplus femur-rubrum* De Geer) and meal worm larvae. Six individuals were used for each treatment with a corresponding number of controls. After the insects were killed by subsection to various treatments, as described later, they were thoroughly rinsed in acetone and dissected under 95 per cent alcohol. The controls were killed by decapitation.

Dissections were made with the aid of a binocular dissecting microscope. The insect was pinned to the waxed bottom of a dissecting dish and a longitudinal incision was made ventrad of the spiracles with a curved scissors. The cut surface was pinned back and the alimentary canal pushed aside exposing the ventral nerve cord. By carefully teasing the cord away from its surrounding tissues it was possible to lift it intact from the floor of the abdomen. For this purpose dental lances were found to be very useful. Dissections of the head were performed by making incisions through the compound eyes and following the optic nerves to their source in the lobes of the brain. When the brain was located with certainty its removal was easily effected. The suboesophageal ganglion was reached by an incision through the ventral surface of the head after first removing the labium and maxillae. Its obvious relation to the oesophagus aids in its location.

The brain, suboesophageal ganglion, and thoracic ganglia were fixed separately in 95 per cent alcohol for 16 hours. The ventral nerve cord and abdominal ganglia were fixed in a similar manner. The tissue was stained for five and one-half hours with 0.1 per cent aqueous toluidine blue, following a technique attributed to Nissl by Krause (9, v. 3, p. 651) used in medicine for the detection of paralysis in humans. The tissue was washed in 95 per cent alcohol, dehydrated in absolute alcohol, and imbedded in paraffin after running through xylol. As the tissue was very fragile, gradual transitions were made by using combinations of xylol and alcohol and paraffin and xylol. Sections of 5 μ thickness were made.

RESULTS

EFFECT ON NERVES

Pyrethrins

Adult grasshoppers and meal worm larvae were killed by applying pyrethrum concentrates on the dorsal surface. The concentrates were prepared according to a method of extraction previously published (21) and contained 80 per cent or more of pyrethrin I and II. The insects were treated for 16 hours to assure death. They were then dissected and pre-

pared for histological study as described in the preceding paragraph. Cross sections of this nerve tissue stained blue throughout in the controls. Scattered among the blue-staining cells, in the nerve tissue from insects killed by the pyrethrins were areas that stained violet and other areas that were vacuolated, the margins of which stained dark blue. Lesions of this general description were found in the brain, suboesophageal ganglion, thoracic ganglia, abdominal ganglia, and connectives.

Deeply stained masses in the protoplasm of neurons (Nissl granules) are termed tigroid. It has been suggested by Szent-Györgyi (19) that the tigroid is a reserve polysaccharide of the nerve cells similar to glycogen. The term tigrolysis has been used in the medical literature to describe pathological degeneration of the Nissl granules.

Brain: The cortical region showed acute cellular degeneration in the adult grasshopper. There was marked tigrolysis and vacuolization of the tissue. The degenerated tissue which stained violet appears black in the photomicrograph as contrasted with the uniform blue-stained tissue of the controls. (Fig. 1, compare A and B.)

Suboesophageal ganglion: The cellular degeneration in the adult grasshopper is less marked than in the brain. The cortical region showed violet-stained areas. Vacuolization and tigrolysis were less marked than in the brain. (Fig. 2, compare A and B.)

Thoracic ganglia: The dorsal portion of the cortical region in the adult grasshopper showed acute degeneration. There were dense masses of violet-stained tissue which appears black in the photomicrograph as contrasted with uniform blue-stained tissue of the controls (Fig. 3, compare A and B). Tigrolysis and vacuolization of the tissue were less marked than in the brain.

Abdominal ganglia: The controls in the meal worm larvae showed a rather homogeneous texture that stained uniformly blue throughout. In ganglia of larvae killed by pyrethrum concentrate the cortical region stained violet and there were marked vacuolization of the tissue and tigrolysis.

Connectives: The degeneration of the tissue in both the adult grasshopper and meal worm larva was less marked than in the ventral ganglia with occasional violet-stained areas as contrasted with the light uniform blue-stained tissue of the controls.

Comparison with Triorthocresyl Phosphate

Triorthocresyl phosphate is known to cause nerve lesions in higher animals. Its effects on nerve tissue have been the subject of an extensive study by Smith and coworkers (11, 16, 17, 18) of the United States Public Health Service prompted by the discovery that the wave of so-called "ginger paralysis" which occurred in many sections of the United States

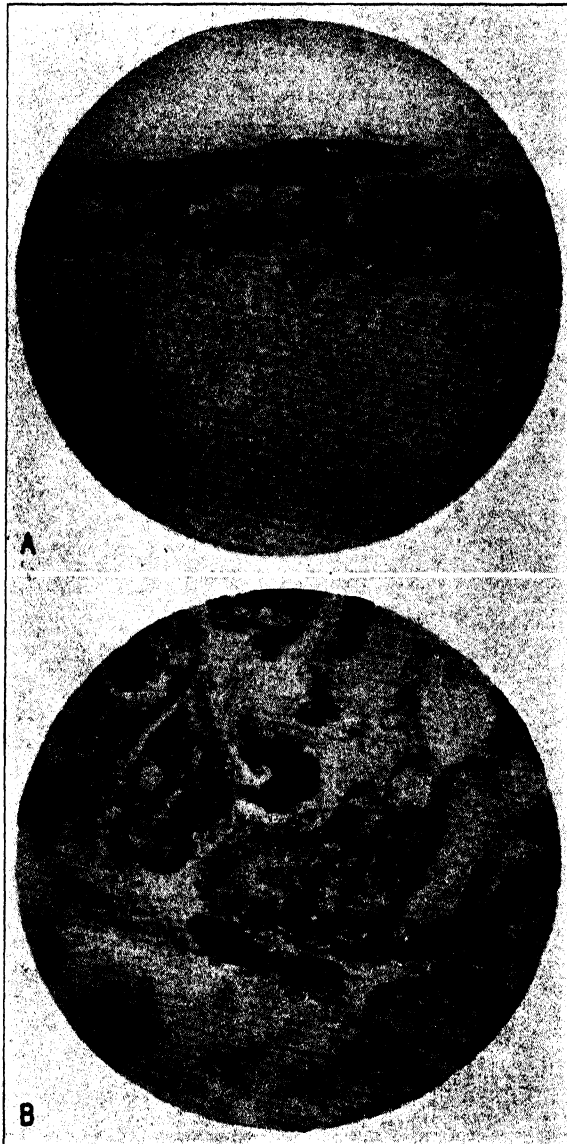


FIGURE 1. Effect of pyrethrum concentrate on grasshopper (*Melanoplus femur-rubrum*) brain. Cross sections of cortical regions of the brains of adults stained with toluidine blue. $\times 1300$. (A) Killed by decapitation; (B) Killed by pyrethrum concentrate. Note the disintegration of the tissue; the violet-stained areas appear black in the photomicrograph.

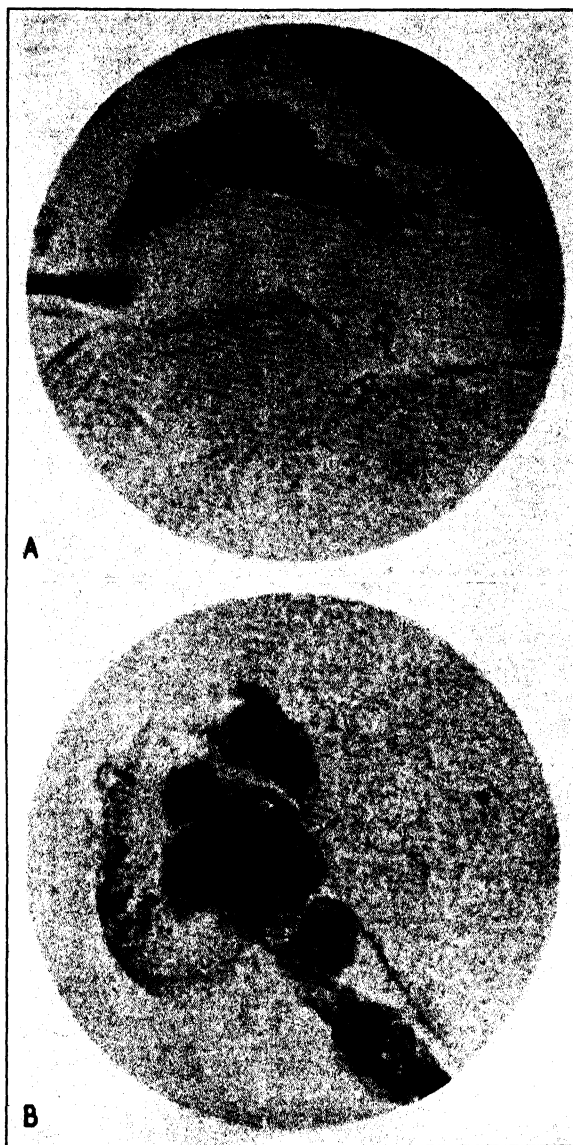


FIGURE 2. Effect of pyrethrum concentrate on grasshopper (*Melanoplus femur-rubrum*) subesophageal ganglion. Cross sections of subesophageal ganglia of adults stained with toluidine blue. $\times 1300$. (A) Killed by decapitation; (B) Killed by pyrethrum concentrate. The disintegration of the tissue is less marked than in the brain; the violet-stained areas appear black in the photomicrograph.

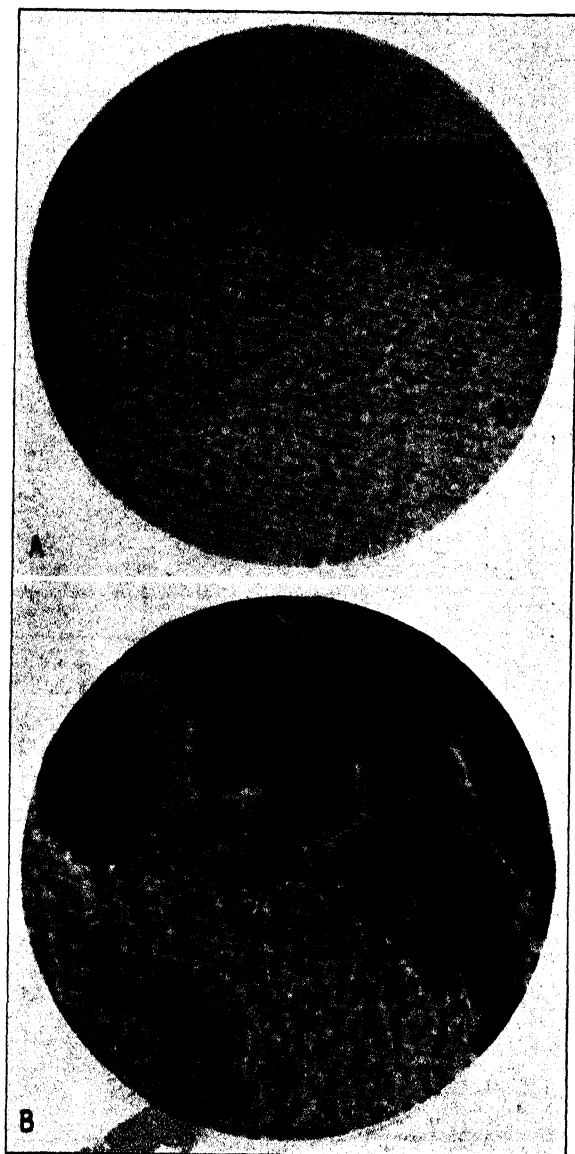


FIGURE 3. Effect of pyrethrum concentrate on grasshopper (*Melanoplus femur-rubrum*) thoracic ganglion. Cross sections of cortical region of thoracic ganglia of adults stained with toluidine blue. $\times 1300$. (A) Killed by decapitation; (B) Killed by pyrethrum concentrate. Note the acute disintegration of the tissue; the violet-stained areas appear black in the photomicrograph.

in 1930 was due to this compound or to a related compound which had been used as an adulterant. The phenol esters were shown to possess an affinity for nerve cells in higher animals, particularly for motor nerve cells. Orthocresol was found to attack the myelin of the conducting nerve fibers either peripherally as phosphate of orthocresyl or centrally as well as peripherally as phosphite of orthocresyl.

The effect of triorthocresyl phosphate on the nervous system of insects was considered of interest in view of its known paralytic action on man and higher animals although this property precludes its use as an insecticide. Meal worm larvae were killed by smearing them with this compound. After the larvae were dead they were carefully rinsed in acetone solution and the ventral nerve cord removed, fixed, stained, and sectioned as described above. Care must be exercised in making dissections of treated insects with this compound that there are no abrasions on the hands as the amount sufficient to cause paralysis when absorbed by the blood stream is known to be very small. The lesions found in the ventral ganglia of meal worm larvae with this compound resembled those produced by the pyrethrins. There are vacuolization of the tissue and tigrolysis (Fig. 4, compare A and B).

Rotenone

An examination of nerve tissue of insects killed by rotenone failed to show any appreciable lesions when stained with toluidine blue. Recent work by Haag (5) on higher animals indicates that the most characteristic effect of rotenone is on respiration, death resulting due to respiratory failure.

High Radio Frequency and Heat²

It is generally known that vacuum tubes generate an electric magnetic field composed of many different wave lengths. Radio broadcasting is only one application of this principle. The use of short radio waves in the therapeutic field and in the extermination of insects has been a more recent development. Whitney (20) found that insects and other animals could be easily killed in an intense radio field, but only after evidence of over-heating, so that death was apparently due to heat. Studies on the effect of the radio field on salt solutions, particularly solutions similar to blood, by Hosmer (6) showed that effective heating occurred in a range of alternating frequencies of the order of 50 to 10 million cycles, or 6 to 30 meters.

Recently Donnelly (4) has called attention to the fact that the heating of a solution varies with the specific electric conductivity. This principle is also made use of in raising the body temperature of animals by Knudson and Schaible (8) and is applied by Carpenter and Page (3) in therapeutics

² The writer is indebted to Mr. H. St. Laurent for cooperation in this phase of the work.

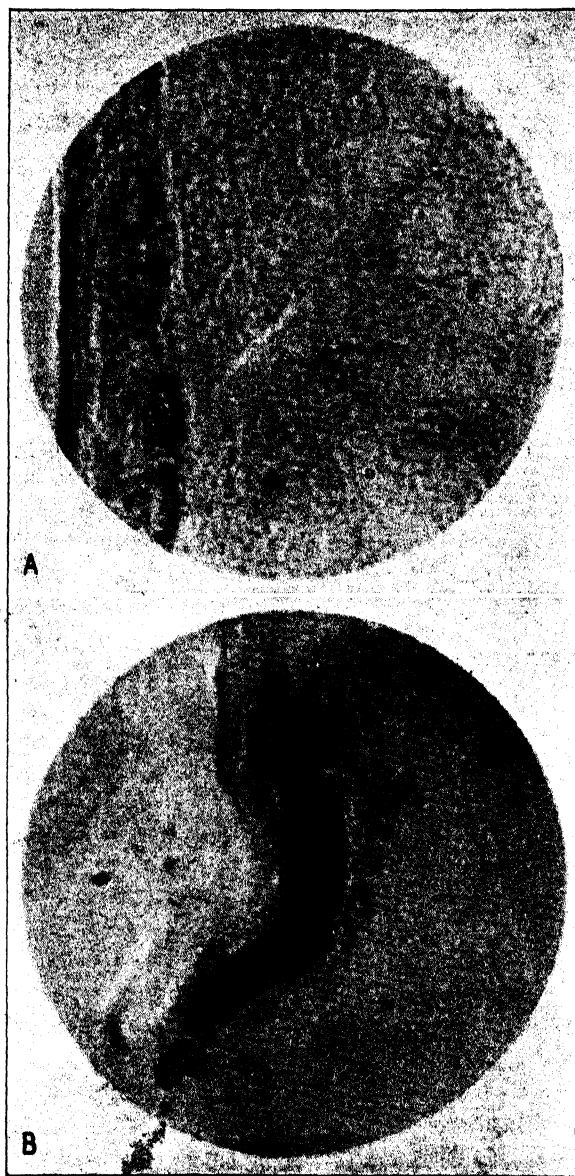


FIGURE 4. Effect of triorthocresyl phosphate on meal worm larva (*Tenebrio molitor*) ventral nerve ganglion. Cross sections of ventral nerve ganglia of meal worm larvae stained with toluidine blue. $\times 1300$. (A) Killed by decapitation; (B) Killed by triorthocresyl phosphate. Note vacuolated tissue of lesion; the violet-stained areas appear black in the photomicrograph.

in combating certain diseases in which excessive body temperatures may alleviate or even cure the disease. Schliephake (15) has reported that definite nerve cells were destroyed in the brain of rabbits when subjected to wave lengths of 4 meters or less, while adjacent cells remained unharmed.

It was found during this investigation that it was possible to kill meal worm larvae by placing them for a period of two minutes in a high radio frequency field of approximately 300,000,000 cycles or approximately 1-meter wave length and of 2.9 amperes. Alternating current was used on the anodes of an oscillator comprising two 75-watt type 852 tubes in a push-pull circuit. The field was applied by means of two small metallic electrodes (2.5×3 inches) two inches apart. The tank circuit comprised a single turn of copper tubing. The electrodes were fed inductively by means of a pick-up coil. The circuit comprising the pick-up coil and the electrodes was made to resonate with the tank circuit of the oscillator.

By means of thermocouples it was found that the temperature of the body cavity of larvae killed by this means did not exceed 45°C ., which is below the thermal death point of these insects for heat applied externally for a similar period. The ventral ganglia were removed by dissection after exposure to high radio frequency of the magnitude indicated above and fixed, stained, and sectioned as described in the section entitled "Material and Methods." It was found that the ganglia would not take the stain appreciably while the controls stained uniformly blue throughout. Microscopic preparations gave the impression that there had been very rapid solution of the cells. (Compare Fig. 4 A with Fig. 5 A.) A similar effect was noted by Schereschewsky (14) in mouse sarcoma. In order to determine whether this effect was the same as that produced by heat a comparison was made of the ventral ganglia of meal worm larvae that had been killed by heating for 30 minutes at 52°C . This tissue also failed to stain appreciably, but there was no evidence of solution of the cells, but rather an indication of coagulation. (Compare Fig. 4 A with Fig. 5 B.) This suggests that high radio frequency may produce a different effect on nerve cells than heat applied externally.

While earlier workers dealing necessarily with comparatively longer wave lengths attributed the effects of high radio frequency on animals to heat, more recent work by Oettinger and others (1, 12) has lent support to the view that selective frequency may be of more importance than heat when very short wave lengths are involved. Our preliminary results on insects indicate that the effect differs from that obtained with lethal temperatures applied externally.

EFFECT ON MUSCLES

Krüger (10) reported morphological changes in the muscles, hypodermis, and nerves of *Corethra plumicornis* larvae killed by pyrethrum. It

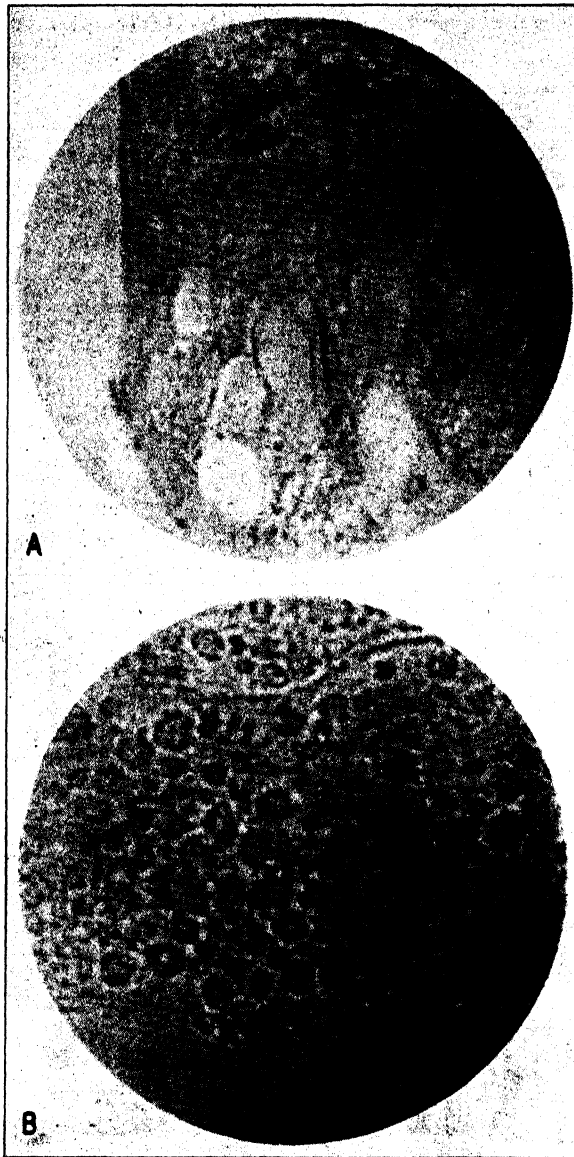


FIGURE 5. Effect of high radio frequency and heat applied externally on meal worm larva (*Tenebrio molitor*) ventral nerve ganglion. Cross sections of ventral nerve ganglia of larvae stained with toluidine blue. $\times 1300$. (A) Killed by high radio frequency (300,000,000 cycles, 1 meter, 2.9 amperes). The tissue failed to stain. Note the large vacuoles suggesting solution of the cells; compare with Figure 4 A; (B) Cross section of nerve ganglion of larva killed by heat ($52^{\circ}\text{C}.$) applied externally. $\times 1300$. The tissue failed to stain. Note that the tissue is not vacuolated and there is evidence not of solution but of coagulation of the cells. Compare with Figure 4 A.

would be of considerable interest to compare the pharmacological action of the pyrethrins on insect muscle with that of the muscles of higher animals. The very small size of insect muscles and the absence of a muscle sheath makes such a comparison very difficult with insects, so that this study was confined to frog's muscle. As very little has been published regarding the classification of the pyrethrins pharmacologically, an attempt was made to investigate their action on frog's gastrocnemius muscle following a slightly modified technique,³ described by Howell (7 p. 23-33),

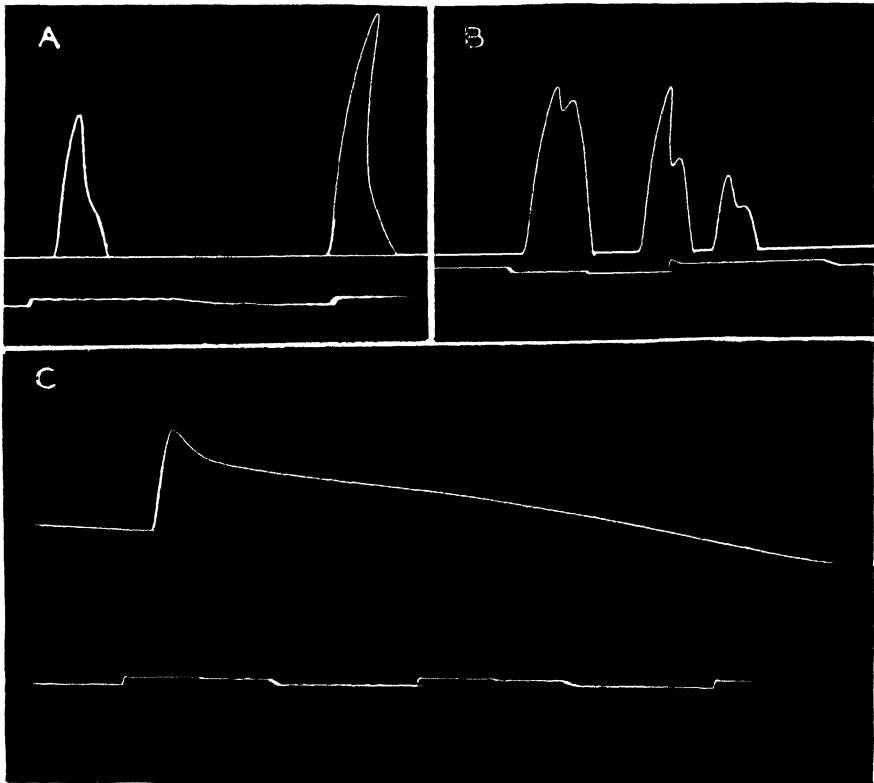


FIGURE 6. Comparison of pyrethrum concentrate and veratrine on frog's gastrocnemius muscle contraction. (A) Curve of normal muscle contraction; (B) Curve of pyrethrin-treated muscle contraction; (C) Curve of veratrine-treated muscle contraction. (All natural size.)

used in the study of narcotics. A 0.5 cc. concentrate containing approximately 80 per cent of pyrethrin I and II was injected by means of a hypo-

³ The writer is indebted to Dr. C. S. Leonard, Director of the Experimental Research Laboratories of Burroughs Wellcome and Company, Inc., and to members of its staff for their kind coöperation.

dermic needle into the lymph sac of a pithed frog. The muscle contractions were stimulated by means of an induction coil and recorded on the drum of a kymograph. The curve produced by a pyrethrin-treated muscle was found to be of a definite type (Fig. 6), showing a slight secondary contraction after partial relaxation. The relaxation time was somewhat greater than that of a normal muscle and considerably shorter than that of a veratrine-treated muscle (0.5 cc. saturated solution injected into the lymph sac).

SUMMARY

1. Nerve lesions have been found in meal worm larvae (*Tenebrio molitor*) and adult grasshoppers (*Melanoplus femur-rubrum*) that had been killed by pyrethrum concentrates applied externally following a technique used in the detection of paralysis in humans which involves the use of toluidine blue.

2. Lesions were found throughout the main parts of the central nervous system, in the brain, the suboesophageal ganglion, thoracic ganglia, abdominal ganglia, and in the connectives of the above named species.

3. From a study of the histopathology of the nerves of insects killed by the pyrethrins one is led to conclude that death is caused by the destruction of the cells of the central nervous system accompanied by paralysis.

4. Lesions were found in the ventral nerve ganglia of meal worm larvae killed by triorthocresyl phosphate.

5. No destruction of nerve tissue comparable to that caused by the pyrethrins was found in the above named species of insects killed by rotenone.

6. Meal worm larvae killed by exposure for two minutes in a high radio frequency field of approximately 300,000,000 cycles or approximately 1 meter and of 2.9 amperes showed nerve lesions unlike those produced in larvae killed by heat (52° C.) applied externally.

7. The pharmacological action of the pyrethrins on frog's gastrocnemius muscle was studied. It was found that the curve produced by a pyrethrin-treated muscle appeared to be of a definite type and the relaxation time was somewhat greater than that of a normal muscle response and considerably shorter than that of a veratrine-treated muscle.

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SOME EFFECTS OF RADIATION FROM A QUARTZ MERCURY VAPOR LAMP UPON THE MINERAL COMPOSITION OF PLANTS

W. D. STEWART AND JOHN M. ARTHUR

INTRODUCTION

Since the discovery that animals on a rickets-producing diet were protected against rickets by irradiation, the rôle of irradiation in animal nutrition has been the object of much excellent experimentation. As a result of the earlier of these investigations it is now well known that the radiant energy effective in the protection of animals against rickets exerts its influence indirectly by activation of the ergosterol in the skin. More recent investigations have shown that small doses of irradiated ergosterol given to rachitic animals bring about increased retention of calcium and phosphorus by the bone, healing of rachitic lesions, and increase in bone ash, whereas overdosage causes hypercalcemia, hyperphosphatemia, or both, of the blood serum, removal of calcium and phosphorus from the bone, deposition of calcium and phosphorus in the soft tissues, and increased excretion of these two elements. Further, the calcium and phosphorus level and the ratio of calcium to phosphorus in the diet were found to be very important factors in determining the type of response to overdosage of irradiated ergosterol.

Little is known of the changes in plants resulting from irradiation. Plants grown in the fall, winter, and early spring seem to be of no value as food for the protection of rats against rickets. Even when grown in the summer plants appear to have little or no anti-rachitic value; but, upon irradiation from a quartz mercury vapor arc or other ultra-violet source, the tissues are rendered potent. Not all plants, however, possess this property even upon prolonged irradiation; cabbage has been shown to be an example of such plants. Experimental evidence of changes in mineral composition of plants induced by irradiation is scanty and inconsistent. The work from which this evidence is drawn is to be discussed later. The experiments described in this paper had as objectives: (A) A study of the mineral composition of plants as affected by irradiation under a quartz mercury vapor arc; (B) An investigation of the calcium and phosphorus content of irradiated and non-irradiated plants; (C) Comparison of response by plants under varying light intensity; (D) The explanation of discrepancies in the results reported by previous investigators; (E) Evidence to show whether or not response to irradiation by the plant might be attributed to activation of ergosterol present in the plant.

MATERIALS AND METHODS

Species used. The plants used were: tobacco (*Nicotiana tabacum* L. var. Turkish), salvia (*Salvia splendens* Ker.), tomato (*Lycopersicon esculentum* Mill. var. Bonny Best), lettuce (*Lactuca sativa* L.), cabbage (*Brassica oleracea* L. var. *capitata* L.), Jimson weed (*Datura stramonium* L.).

Cultural procedure. Plants for some of the experiments were seeded and grown in flats containing soil; for some, seedlings were transplanted into pots filled with soil or sand; for others, cuttings were made, rooted in various media, and grown in soil or nutrient solutions. Solutions for water culture were made up according to the directions given by Guthrie (8). When plants were grown in nutrient solutions the cultures were aerated constantly by passing a stream of washed oxygen bubbles through the liquid in the cylinders. The solutions were changed once each 48 hours. Plants were grown for one week under the conditions of the experiment before irradiation to permit acclimatization, and were usually five to seven inches in height at the time of the first irradiation.

Irradiation. The plants were placed under a Cooper Hewitt Uviarc arc lamp, used and described by Arthur (1), 15 inches from the light source and irradiated. Plants were irradiated under the open arc and through glass filters. The energy output of the lamp, the transmission graphs for the filters used, and the characteristics of the lamp were the same as those published by Arthur (1).

Sampling. At the conclusion of an experiment plants were either separated into leaves and stems (the petiole being included in the stem fraction), or else the entire plant was harvested. The green weight was taken, and the plants were air-dried.

Chemical analyses. The material was ground in a "Nixtamal" mill, mixed, placed in weighing bottles, and dried overnight at 100° C. Portions were then weighed out, by difference in weight before and after removal of a fraction of the material, into tared ashing capsules. The material was moistened with a mixture of equal parts of alcohol and glycerine, charred and then ashed in a muffle furnace at 650° C. for 16 hours. All samples for comparison were ashed at the same time, in the same furnace. The percentage of ash and of other constituents was computed from the weight of dry material used. The ash was dissolved in nitric acid and transferred with water to volumetric flasks.

Calcium, magnesium, and phosphorus were determined from aliquots according to the procedure of Tisdall and Kramer (17). Separate ash fusions with potassium bisulphate were made for the manganese analyses, and the standard periodate method was employed to ascertain the amount of the element present.

TABLE I
ASH ANALYSES OF IRRADIATED AND NON-IRRADIATED PLANTS; ALL EXPOSURES 15 INCHES FROM THE
UNSHIELDED QUARTZ TUBE AT 48-HOUR INTERVALS
A. Plants grown in flats

Plant	Date of sampling	Irradiation Exposure		Part analyzed	% ash		% calcium		% P ₂ O ₅	
		Sec.	No.		Irr.	Check	Irr.	Check	Irr.	Check
Lettuce	Dec. 1928	30	10	Ent.	25.9	23.5	1.73	1.46	2.36	2.02
Lettuce	Feb. 1929	30	2	Ent.	24.3	20.3	1.54	1.37	2.04	1.87
Lettuce	Mar. 1929	30	10	Ent.	30.0	24.7	1.20	1.24	1.79	1.54
Lettuce	May 1929	30	10	Ent.	35.7	31.7	2.03	1.88	1.41	1.19
Tomato	July 1929	30	10	Lvs.	15.7	13.8	2.28	2.15	0.96	0.82
Tomato	July 1929	60	10	Lvs.	17.2	13.8	2.05	2.15	1.28	0.82
Tomato	July 1929	30	10	Lvs.	15.3	14.5	2.60	2.12	1.14	0.66
Tomato	July 1929	60	10	Lvs.	18.4	14.5	2.53	2.12	1.47	0.66
Cabbage	Feb. 1929	30	2	Ent.	21.5	21.3	3.57	3.59	1.53	1.42
Cabbage	July 1931	15	6	Ent.	20.0	21.0	3.40	3.50	1.06	1.23
Tomato	Mar. 1930	30	3	Ent.	15.1	13.8				
Tomato	Mar. 1930	60	3	Ent.	17.9	13.8				

B. Plants grown in pots

Plant and No. used	Date of sampling, 1931	Irradiation		Part analyzed	Dry wt., g.		% ash		% calcium		% P ₂ O ₅	
		Exposure	No.		Irr.	Check	Irr.	Check	Irr.	Check	Irr.	Check
Tomato 72	Apr.	5	8	Lvs. Stms.	35.0 41.0	30.2 39.0	19.1 19.9	18.2 18.5	6.72 3.52	6.60 3.20	0.86 0.50	0.99 0.58
Cabbage 40	Mar.	15	6	Lvs. Stms.	75.0 17.0	132.0 22.0	16.9 12.7	19.2 15.8	6.77 1.89	8.43 2.33	0.74 1.30	0.84 1.36
Cabbage 60	July	15	15	Lvs. Stms.	212.0 42.0	242.0 42.0	12.8 10.2	12.9 10.6	5.40 1.60	5.90 1.75	0.76 1.29	0.80 1.40
Tobacco 52	Mar.	15	12	Lvs. Stms.	19.2 5.6	30.0 14.0	19.8 17.8	19.1 15.8				
Tobacco 30	Feb.	10	6	Lvs. Stms.	25.0 12.2	31.0 13.7	12.0 11.4	11.3 10.1				
Tobacco 60	July	15	14	Lvs. Stms.	155.0 125.5	171.0 155.6	22.4 20.3	20.7 17.2				

RESULTS

PLANTS GROWN IN THE GREENHOUSE, AND IRRADIATED DURING PERIODS OF LOW LIGHT INTENSITY

Irradiation under open arc. Earlier attempts in this laboratory to find differences in ash content and mineral constituents of irradiated and non-irradiated plants were not successful; sometimes the irradiated plants showed an increase, at other times no increase or even a decrease. Later during a period of high light intensity, it was observed that plants irradiated immediately after an exposure of 48 hours to cloudy weather displayed the "shine" characteristic of injury under the arc and exhibited an increase in ash, whereas plants irradiated after an equal exposure to non-cloudy weather gave no indication of "shine" or of change in ash content. This was true whether the plants were irradiated for 10 or 15 seconds, 15 inches from the open arc—dosages that have marked injurious effects upon plants grown in the dark.

All plants indicated in Table I were irradiated under the open arc. The exposures varied from 5 to 60 seconds, repeated at 48-hour intervals until the plants had received from two to fourteen irradiations. The plants in these experiments received at least two irradiations following periods of 36 to 48 hours of cloudy weather. Those plants irradiated eight to ten times had at least three irradiations following such cloudy periods. Lettuce, tomato, tobacco, and cabbage plants used in the tests were grown in either flats or pots containing a well fertilized soil.

Results from these experiments are given in Table I. Irradiated plants, with the exception of cabbage, were higher in ash than non-irradiated. The increase in ash occurred whether the computation was on the basis of dry weight of stem, leaf, or of the entire plant. Associated with the increase in ash was an increase in calcium or phosphorus, or both. Cabbage showed either a decrease or no change in ash with irradiation; the calcium and phosphorus contents were either unchanged or decreased. Magnesium content of all plants was unaltered by irradiation.

Irradiation through glass filters. In order to determine the wave length ranges effective in producing change in mineral composition of plants, glass filters with varying transmission in the ultra-violet were used. Filters were chosen which absorb all or part of the region of wave length shorter than 2900 Å, known to be injurious to plant cells, in order to determine whether an increase in ash resulted from injury to the plant. The following Corning glass filters were used: filter A-1.9 mm. in thickness which transmits one per cent of the incident radiation at wave length 249.7 mμ and has an extreme limit of transmission at 237 mμ; filter C-3.99 mm. which transmits one per cent at wave length 273.7 mμ and has an extreme limit at 235 mμ; and filter F-5.06 mm. which transmits one per cent at wave

TABLE II
ASH ANALYSES OF IRRADIATED AND NON-IRRADIATED PLANTS; ALL EXPOSURES 15 INCHES FROM ARC THROUGH GLASS FILTERS AT 48-HOUR INTERVALS

Plant and No. used	Date of sampling	Irradiation			Part analyzed	Dry wt., g.		% ash		% calcium		% P ₂ O ₅	
		Filter used	Exposure, min.	No. of exposures		Irr.	Check	Irr.	Check	Irr.	Check	Irr.	Check
Tomato 30	Apr. '31	F	15	8	Lvs. Stms.	49.0 35.0	48.0 37.0	21.9 17.8	19.4 17.0	8.83 4.54	8.55 4.41	1.17 0.82	1.07 0.68
Tobacco 40	Apr. '31	F	15	11	Lvs. Stms.	64.0 17.0	82.0 25.0	23.3 24.9	22.8 23.1	6.68 2.38	3.70 2.04	1.41 1.07	1.48 0.99
<i>Datura</i> 16	May '31	F	10	14	Lvs.	20.0	20.5	13.9	13.6	1.68	1.60	1.32	1.14
Tomato 10	Oct. '30	C	60	8	Ent.	18.0	18.1	19.6	18.0	2.51	2.18	1.30	1.25
Tomato 10	Oct. '30	C	120	8	Ent.	17.9	18.1	20.6	18.0	2.51	2.18	1.30	1.25
Tomato 10	Oct. '30	C	180	8	Ent.	18.2	18.1	21.0	18.0	2.84	2.19	1.60	1.25
Tomato 16	July '29	A	5	10	Lvs.	19.1	19.2	17.3	13.8	2.55	2.15	0.85	0.82
Tomato 16	July '29	A	15	10	Lvs.	18.9	19.2	17.8	13.8	2.53	2.15	1.11	0.82
Tomato 22	July '29	A	5	10	Lvs.	23.8	24.0	19.3	14.5	2.89	2.12	0.92	0.66
Tomato 22	July '29	A	15	10	Lvs.	22.1	24.0	18.7	14.5	2.95	2.12	0.96	0.66
Cabbage 27	Feb. '30	A	5	10	Ent.	7.8	9.4	21.4	21.3	3.58	3.59	1.48	1.42
Cabbage 27	Feb. '30	A	15	10	Ent.	6.5	9.4	21.5	21.3	3.56	3.59	1.40	1.42
Tomato 18	Aug. '33	W.G.*	15	1**	Lvs. Stms.	9.0 4.2	8.9 4.5	15.2 27.7	15.2 28.3	2.73 1.61	2.78 1.74	1.26 1.16	1.28 1.32
Lettuce 56	Feb. '29	A	5	2	Ent.	7.0	6.8	22.3	20.3	1.57	1.37	1.96	1.87
Lettuce 56	Feb. '29	A	15	2	Ent.	7.9	6.8	21.7	20.3	1.49	1.37	2.07	1.87
Tomato 32	Nov. '33	W.G.*	5	3	Lvs. Stms.	8.1 5.3	8.5 5.5	15.5 26.6	15.9 26.2	3.92 2.90	4.06 2.77	0.95 0.65	0.96 0.76
Tomato 32	Nov. '33	W.G.*	10	3	Lvs. Stms.	7.8 5.2	8.5 5.5	16.0 26.3	15.9 26.2	4.01 2.75	4.06 2.77	1.04 0.79	0.96 0.76
Tomato 32	Nov. '33	W.G.*	15	3	Lvs. Stms.	7.9 5.2	8.5 5.5	16.1 25.9	15.9 26.2	4.00 2.79	4.06 2.77	0.99 0.74	0.96 0.76

* Window glass.

** Sampled 72 hrs. after irradiation.

TABLE III
ASH ANALYSES OF IRRADIATED AND NON-IRRADIATED PLANTS; ALL EXPOSURES 15 INCHES FROM UNSHIELDED QUARTZ TUBE; INTERVAL BETWEEN TIME OF IRRADIATION AND RESPONSE BY PLANT

Plant	Date of sampling	Treatment	Dry wt., g.		% ash		% calcium		% P ₂ O ₅	
			Irr.	Check	Irr.	Check	Irr.	Check	Irr.	Check
A. Irradiation by "twin-leaf" method										
Salvia	June '31	Irradiated once for 30 sec.; sampled 48 hrs. after irradiation.	6.2	7.1	11.3	11.2				
Tomato	June '30	Irradiated once for 30 sec.; sampled 72 hrs. after irradiation.	4.8	5.8	10.8	10.0				
Tomato	June '30	Irradiated once for 30 sec.; sampled 72 hrs. after irradiation.	2.9	3.1	9.9	9.3				
Tomato	June '30	Irradiated twice for 30 sec. at 48-hr. interval; sampled 120 hrs. after first irradiation.	2.4	2.9	18.1	16.9	3.42	2.96	0.96	0.92
Tomato	June '30	Irradiated four times for 20 sec. at 48-hr. interval; sampled 300 hrs. after first irradiation.			16.5	15.4	4.68	4.24	0.92	0.80

TABLE III (continued)
B. Entire plant irradiated once, sampled 48 hrs. later

Tomato	Sept. '33	Irradiated 15 sec. 30 plants.	Leaves Stems	14.9 8.9	15.4 7.7	14.9 26.8	15.6 28.7	2.85 1.70	2.91 1.79	1.41 1.16	1.40 1.16
Tomato	Sept. '33	Irradiated 30 sec. 30 plants.	Leaves Stems	16.4 9.4	15.4 7.7	15.6 27.7	15.6 28.7	2.94 1.75	2.91 1.79	1.38 1.15	1.40 1.16
Tomato	Sept. '33	Irradiated 60 sec. 30 plants.	Leaves Stems	14.6 7.8	15.4 7.7	15.4 27.8	15.6 28.7	3.01 1.75	2.91 1.79	1.41 1.16	1.40 1.16
Tomato	Sept. '33	Irradiated 120 sec. 30 plants.	Leaves Stems	14.3 8.4	15.4 7.7	15.8 27.5	15.6 28.7	3.13 1.90	2.91 1.79	1.52 1.33	1.40 1.16

C. Entire plant irradiated once, sampled 72 hrs. later

Tomato	Sept. '33	Irradiated 15 sec. 28 plants.	Leaves Stems	9.2 4.5	9.2 4.5	16.8 28.2	16.2 29.0	2.97 1.91	2.85 1.90	1.34 1.21	1.35 1.23
Tomato	Sept. '33	Irradiated 30 sec. 28 plants.	Leaves Stems	8.0 4.0	9.2 4.5	16.5 28.1	16.2 29.0	3.02 1.97	2.85 1.90	1.45 1.42	1.35 1.23
Tomato	Sept. '33	Irradiated 60 sec. 28 plants.	Leaves Stems	7.0 4.0	9.2 4.5	16.4 27.3	16.2 29.0	3.08 1.97	2.85 1.90	1.56 1.47	1.35 1.23
Tomato	Sept. '33	Irradiated 120 sec. 28 plants.	Leaves Stems	7.0 4.0	9.2 4.5	17.8 26.5	16.2 29.0	3.22 1.98	2.85 1.90	1.68 1.59	1.35 1.23

length 293.5 $m\mu$ and has a transmission limit at 286 $m\mu$. Also plants were irradiated through window glass having a limit of transmission at 313 $m\mu$.

Data from irradiation of plants through these filters, in Table II, revealed the same type of response by the plant to irradiation as under the open arc except when window glass was used. Cabbage again failed to give an increase in ash, calcium, or phosphorus with irradiation. The other species showed an increase in ash accompanied by an increase in calcium or phosphorus or both, as a result of irradiation of wave lengths shorter than 313 $m\mu$. Irradiation through filter F transmitting only to 286 $m\mu$ was equally as effective as that through filters transmitting the shorter wave lengths in the ultra-violet. No visible injury was produced through filter F but marked injury through filter A. There was no relationship between injury to the plant and response to irradiation. Irradiation through window glass failed to produce a response—no increase in ash, calcium, or phosphorus. Magnesium content apparently was unaffected by irradiation. The most interesting observation to be had from these experiments was that the wave lengths effective in producing an increase in ash and altering the calcium-phosphorus level of plants were in the same region as those involved in the activation of ergosterol and the production of a similar response in rachitic animals. Almost equally interesting was the fact that cabbage failed to show any increase in ash, calcium, or phosphorus. This plant displayed the same lack of response under the open arc.

Irradiation of plants to determine interval between time of irradiation and response. Plants for these experiments were grown in four-inch pots. In Table III A are the results from tests by the "twin-leaf" method as used by Denny (4). Following this method, one leaflet of tomato was irradiated, the opposite leaflet wrapped with tinfoil at the time of irradiation serving as a control; with salvia, opposite leaves were used in the same manner. Only the first six leaves from the tip of each plant were used. Leaves irradiated once for 30 seconds gave an increase in ash 72 hours later. The tests furnished more evidence of an increase in ash with irradiation as well as an increase in calcium or phosphorus, or both. With this procedure it appears that the response to irradiation in the leaf was restricted to the portion irradiated since the irradiated leaf maintains its increase in ash and altered calcium-phosphorus level 300 hours after the first irradiation over that of its opposite and adjacent leaflet. The magnitude of the response was as great as though leaves from different plants were used.

Since the "twin-leaf" method gave no information as to changes in mineral composition of the stem another group of tests was made using the entire plant for irradiation. The results from these experiments in Table III B and C indicated no response to irradiation with a single treatment

of 15, 30, or 60 seconds, 48 hours after irradiation. With a treatment of 120 seconds, however, irradiated leaves showed an increase in ash and this increase in ash was accompanied by an increase in calcium and phosphorus. The stems of these plants were characterized by decreased ash content but an increase in calcium and phosphorus. Seventy-two hours after irradiation all irradiated plants exhibited an increase in ash of the leaves but a decrease in ash content of the stems, the decrease being progressively greater as the dosage increased. Seventy-two hours after irradiation increase in ash of irradiated leaves was accompanied by an increase in calcium with 15 seconds exposure, increase in calcium and phosphorus with exposures of 30, 60, and 120 seconds, the amount of the increase in phosphorus augmenting with increased exposure. Stems of irradiated plants showed no change in calcium or phosphorus content with 15 seconds exposure but an increase in phosphorus that was progressively greater as the dosage increased above 30 seconds. Although the increase in ash of leaves of irradiated plants was less than the decrease in ash of the stems, a translocation of ash constituents from stems to leaves may have taken place. This appears more plausible when one considers that repeated irradiation over a longer period brings about an increase in ash of both leaves and stems.

IRRADIATION OF PLANTS GROWN WITH AND WITHOUT SHADING
CLOTH, UNDER GLASS AND OUTSIDE

Irradiation of plants grown in the greenhouse and under muslin in the greenhouse. Plants were transplanted into four-inch pots filled with soil; one-half of these were placed in the greenhouse, and the other half in a muslin-covered cage in the greenhouse. Air was blown into the greenhouse and into the cage so that temperature differences were low. The glass covering the greenhouse reduced the intensity of outside sunlight approximately 15 per cent. The muslin gave an additional decrease of 65 per cent so that the intensity of the sunlight in the cage was about 30 per cent of that present outside and that of the greenhouse approximately 85 per cent. The plants were grown one week under the conditions stated before irradiation. They were from five to seven inches in height at the time of the first irradiation. All plants were sampled 48 hours after irradiation.

The results from these experiments are given in Table IV. Since these plants were sampled 48 hours after the third irradiation probably no effect of the last irradiation was obtained as it has been shown that an interval of 72 hours was required between time of irradiation and response by the plant unless the dosage was greater than 60 seconds. Although the ultra-violet dosage for the plants in these tests was low the leaves of all irradiated plants were higher in ash than those of the controls. With one exception—plants irradiated during a period of high light intensity in the

TABLE IV

ASH ANALYSES OF IRRADIATED AND NON-IRRADIATED TOMATO PLANTS; ALL EXPOSURES 15 INCHES FROM UNSHIELDED QUARTZ TUBE AT 48-HOUR INTERVAL; PLANTS GROWN IN GREENHOUSE, AND UNDER MUSLIN IN THE GREENHOUSE

Duration of exp. 1932	Treatment	Part analyzed	Dry wt. per plant, g.		% ash		% calcium		% P ₂ O ₅	
			Irr.	Check	Irr.	Check	Irr.	Check	Irr.	Check
Apr. 25 to May 3	G. H. 3 irradi. 20 sec. 20 plts.	Lvs. Stms.	2.3 1.3	2.5 1.4	15.9 21.2	15.2 20.9	3.40 2.73	2.95 2.54	1.28 1.23	1.19 1.26
	Muslin 3 irradi. 20 sec. 20 plts.	Lvs. Stms.	0.7 0.4	0.9 0.5	17.1 24.7	16.9 25.2	3.26 3.02	3.15 2.53	1.70 1.50	1.54 1.32
May 10 to May 18	G. H. 3 irradi. 15 sec. 30 plts.	Lvs. Stms.	2.3 1.5	2.3 1.4	11.9 15.3	11.0 15.4	1.90 1.22	1.93 1.30	0.73 0.68	0.68 0.62
	Muslin 3 irradi. 15 sec. 30 plts.	Lvs. Stms.	1.0 0.6	1.0 0.6	16.1 19.1	15.2 20.0	2.10 1.31	1.92 1.28	1.05 0.82	1.04 0.74
June 3 to June 13	G. H. 3 irradi. 15 sec. 36 plts.	Lvs. Stms.	1.0 0.5	1.3 0.5	14.5 21.4	14.3 22.1	3.24 1.94	2.96 2.09		
	Muslin 3 irradi. 15 sec. 36 plts.	Lvs. Stms.	0.4 0.3	0.5 0.3	17.7 25.2	17.4 25.7	3.22 2.06	3.00 2.10		
May 25 to June 2	G. H. 3 irradi. 10 sec. 30 plts.	Lvs. Stms.	1.2 0.6	1.3 0.6	11.6 16.6	10.9 17.0	2.13 1.73	2.02 1.77	0.62 0.63	0.60 0.55
	Muslin 3 irradi. 10 sec. 30 plts.	Lvs. Stms.	0.6 0.4	0.7 0.4	17.0 23.7	16.1 23.3	2.84 2.15	2.51 1.68	1.06 0.65	0.93 0.71

greenhouse—the increase in ash of leaves of irradiated plants was accompanied by an increase in calcium or phosphorus, or both. The stems of irradiated plants displayed an increase in ash in only two cases, the remaining six showing a decrease. This decrease in ash of the stems of irradiated plants, as was shown in Table III, is a typical early response to irradiation. Three irradiations of 10 to 20 seconds given at 48-hour intervals were insufficient for the production of the increase in ash of both leaves and stems characteristic of plants irradiated over a longer period

TABLE V
ASH ANALYSES OF IRRADIATED AND NON-IRRADIATED TOMATO PLANTS; ALL EXPOSURES 15 INCHES FROM UNSHIELDED QUARTZ TUBE AT 48-HOUR INTERVAL; PLANTS GROWN OUTSIDE, AND OUTSIDE UNDER MUSLIN

Date	Treatment	Part analyzed	Dry wt. per plant (grams)		% ash		% calcium		% P ₂ O ₅	
			Irr.	Check	Irr.	Check	Irr.	Check	Irr.	Check
A. Plants irradiated during period of high light intensity										
Aug. 4, 1932	Outside—in sand; 6 irradiations for 20 sec.; 60 plants	Lvs. Stms.	0.8 0.6	1.0 0.7	12.5 13.6	11.9 13.0	2.68 1.25	2.52 1.17	1.13 1.38	1.05 1.33
	Outside—in soil; 6 irradiations for 20 sec.; 40 plants	Lvs. Stms.	2.3 1.2	2.6 1.5	18.3 21.0	18.9 21.1	3.12 1.91	3.31 1.82	2.08 2.01	2.08 2.03
	Under muslin—in sand; 6 irradiations for 20 sec.; 60 plants	Lvs. Stms.	0.6 0.5	0.8 0.9	19.3 16.4	17.1 15.2	2.44 1.28	2.29 1.00		
	Under muslin—in soil; 6 irradiations for 20 sec.; 40 plants	Lvs. Stms.	1.3 0.9	1.3 1.2	19.3 23.8	18.8 23.8	3.00 1.94	2.93 1.57		
B. Plants irradiated during period of low light intensity										
Sept. 16, 1933	Outside—in sand; 4 irradiations for 20 sec.; 60 plants	Lvs. Stms.	0.7 0.5	0.8 0.5	15.6 17.8	14.9 17.7	2.35 1.22	2.09 1.22	1.49 2.07	1.34 2.05
	Outside—in soil; 4 irradiations for 20 sec.; 40 plants	Lvs. Stms.	0.9 0.9	1.0 0.9	14.6 14.7	12.3 14.1	1.81 1.06	1.67 1.08	0.99 1.88	0.97 1.83
	Under muslin—in sand; 4 irradiations for 20 sec.; 60 plants	Lvs. Stms.	0.5 0.4	0.5 0.5	28.0 27.1	27.5 26.7	2.80 1.34	2.67 1.26	2.32 2.22	2.16 2.07
	Under muslin—in soil; 4 irradiations for 20 sec.; 40 plants	Lvs. Stms.	0.7 0.7	0.8 0.8	25.2 24.8	24.6 24.6	2.66 1.50	2.60 1.43	1.65 1.90	1.49 1.78
June 29, 1933	Outside—in soil; 1 irradiation for 30 sec.; sampled 72 hrs. later; 20 plants	Lvs.			11.3	10.1	1.66	1.28	0.94	0.92
	Under muslin—in soil; 1 irradiation for 30 sec.; sampled 72 hrs. later; 20 plants	Lvs.			13.2	11.8	1.65	1.33	1.16	1.15

at the same interval with similar exposure. Cloudiness varied so much that comparable conditions were not to be expected. Cloudy weather occurred during the period of irradiation for all of the tests except the one from May 10 to May 18. Plants under muslin in the greenhouse suffered more injury than those grown in the greenhouse. In one instance, injury was about the same. In this case the weather was cloudy for almost the duration of the experiment. The magnesium content was not altered by irradiation, nor was the manganese content changed.

Irradiation of plants grown outside, and under muslin outside. Since plants grown under glass do not receive the shorter wave lengths in the ultra-violet from the sun it was thought that plants grown outside might respond differently to irradiation. For these experiments tomato plants were transplanted into six-inch pots filled with unwashed sand, or with soil. The plants growing in sand were given additions of a nutrient solution lacking calcium. Those in soil received no nutrient solution. Tap water was added to the plants in sand until the plants were growing vigorously. Distilled water was then substituted for the tap water during the period of irradiation.

In Table V A are to be found the results of the experiments of the summer of 1932. The plants were irradiated during a period of high light intensity. Irradiated plants outside in sand were higher in ash and calcium than the non-irradiated; those outside in soil showed neither increase in ash nor calcium. Phosphorus content was unaltered for plants in both sand and soil. Irradiated plants under muslin in both sand and soil exhibited increase in ash and calcium. Phosphorus was not determined for the plants under muslin. Plants in sand showed greater injury outside and under muslin than plants in soil. The injury to growth evinced as decrease in height was greater than the injury expressed as decrease in dry weight. This injury was greater for plants grown under muslin, in either sand or soil, than for those grown outside.

The data in Table V B were taken from tests made during the summer of 1933. The plants were irradiated during a period of low light intensity. Under these conditions irradiated plants in both sand and soil were higher in ash than the non-irradiated; in sand the increase in ash was accompanied by an increase in calcium and phosphorus of the leaves, and in soil by an increase in calcium of the leaves. Although only four irradiations were given, the stems of irradiated plants were higher in ash than those of the controls. Under muslin, leaves and stems of plants in sand and soil showed the usual increase in ash with irradiation, and an increase in calcium and phosphorus. Another test, with plants in soil in which only one irradiation of 30 seconds was given, gave an increase in ash for the irradiated leaves, outside and under muslin, when sampled 72 hours after irradiation. The calcium content of the irradiated leaves was higher than that of the con-

trols in each instance with no change in phosphorus. The manganese content of plants used for these experiments was not affected by irradiation.

PLANTS TREATED WITH IRRADIATED ERGOSTEROL

Basal leaves of plants rubbed with olive oil saturated with irradiated ergosterol. The experiments with the irradiation of plants suggested a relationship between response to irradiation and activation of ergosterol. This concept would be strengthened should plants treated with irradiated ergosterol respond in a manner similar to that occurring with the irradiation of plants. Accordingly experiments were made to test the response of plants to irradiated ergosterol.

For these tests ergosterol was taken up in ether, placed in a container, and irradiated under the open arc 35 cm. from the source of light for 35 minutes. While under the lamp the ether solution was stirred constantly. The layer of solution receiving irradiation was about 0.5 cm. in depth. After irradiation the ether was volatilized by passing a stream of air over the solution. The irradiated ergosterol was then washed with water to remove any trace of ether, dried at room temperature, and taken up with olive oil.

The olive oil saturated with irradiated ergosterol was applied to the four basal leaves of tomato plants growing in six-inch pots of soil. Control plants had the four basal leaves rubbed with olive oil only. These basal leaves were rubbed with a glass rod that had been dipped in the material to be applied. Within three to five days after treatment the basal leaves displayed signs of injury. Hence the four basal leaves of both treated and control plants were not included in the material for analysis. For one test the plants were grown three weeks after treatment; for another, the plants were sampled one week after treatment.

The results of these experiments are given in Table VI A. Three weeks after treatment there was an increase in ash of the leaves of plants treated with irradiated ergosterol with no change in ash of the stems. The shoots that grew from the axils of the four basal leaves of the plants treated with the irradiated ergosterol were also higher in ash than those from the axils of the four basal leaves of the plants treated with olive oil only. The calcium content was not altered by the treatment. In the second test, plants treated with irradiated ergosterol showed an increase in ash of both stems and leaves. The change in calcium and phosphorus was not significant for a single experiment. It should be noted that these differences obtained even though the leaves treated with irradiated ergosterol were removed from the plant, and were not included in the material analyzed.

Plants sprayed with olive oil, and with olive oil containing irradiated ergosterol. Plants were grown in four-inch pots of soil; treated plants were sprayed with olive oil containing irradiated ergosterol, and control plants

TABLE VI
ASH ANALYSES OF TOMATO PLANTS TREATED WITH IRRADIATED ERGOSTEROL

Treatment	No. of plants	Part analyzed	Dry wt. per plant (grams)		% ash		% calcium		% P ₂ O ₅	
			Treated	Control	Treated	Control	Treated	Control	Treated	Control
A. Four basal leaves of treated plants rubbed with olive oil saturated with ergosterol; those of controls rubbed with olive oil only										
Rubbed Nov. 17 and sampled Dec. 9, 1932*	20	Leaves Stems Shoots**	3.4 2.3 0.6	3.5 2.3 0.5	19.3 26.9 20.1	18.6 26.8 17.0	2.53 1.81 1.28	2.58 1.74 1.26		
Rubbed Sept. 29 and sampled Sept. 26, 1933*	30	Leaves Stems	1.1 0.7	1.2 0.7	13.9 26.1	13.2 25.9	2.42 1.52	2.31 1.64	0.99 1.23	0.93 1.10
B. Treated plants sprayed with olive oil containing irradiated ergosterol; controls sprayed with olive oil only										
Saturated soln. used; sprayed Jan. 17, sampled Jan. 23, 1934	50	Leaves Stems	0.9 0.4	0.9 0.4	15.6 22.9	13.5 21.3	3.61 3.42	3.26 3.32	0.71 0.59	0.58 0.54
0.1 saturated soln.; sprayed Jan. 23, sampled Jan. 29, 1934	40	Leaves	0.5	0.6	13.4	12.0	3.14	2.64	0.43	0.40

* The 4 basal leaves were not included in the material analyzed.

** Shoots arising from axils of 4 basal leaves.

with olive oil alone. A small atomizer was used for this purpose. The plants were sprayed late in the afternoon. Time of application is important; leaves of plants sprayed earlier in the afternoon take up large quantities of the oil, become translucent, and subsequently are severely injured. This injury was much greater for the oil containing the irradiated ergosterol.

In one test the treated plants were sprayed with olive oil saturated with irradiated ergosterol, and the controls with olive oil alone. The plants were sampled six days later. Leaves and stems of the plants sprayed with olive oil saturated with irradiated ergosterol (Table VI B) were higher in ash than those sprayed with olive oil only. Associated with this increase in ash was an increase in calcium of these tissues.

In another experiment the plants were sprayed with olive oil, and with olive oil containing irradiated ergosterol (1 part of olive oil saturated with irradiated ergosterol to 10 parts of olive oil). The plants were sampled six days later and the sprayed leaves used for analyses. The results of these analyses, in Table VI B, showed an increase in ash of the leaves of the plants sprayed with olive oil containing irradiated ergosterol. The calcium content was also higher for the plants treated with the olive oil containing the irradiated ergosterol than for the plants sprayed with olive oil only.

DISCUSSION OF RESULTS AND LITERATURE

Response to irradiation. Eltinge (5) in 1928 irradiated plants with and without filters, 50 and 100 inches from a quartz mercury vapor arc. From ash analyses of irradiated and non-irradiated plants she obtained results that indicated an increase in ash of plants irradiated under the unscreened lamp. The results, however, were variable, and the increase in ash not always present. Later, in 1931, Fuller (6), using a technique similar to that of Eltinge, irradiated plants nine minutes daily at a distance of 100 inches from the arc through Vita glass over a period of five weeks. He concluded from a single average determination of the ash of irradiated and non-irradiated tomato and cucumber plants that irradiation increases the ash content of these plants. Beeskow (2) reported some findings from preliminary tests on the irradiation of soybean and corn plants. The plants were irradiated at a distance of 39 inches from an unscreened mercury vapor lamp. In the case of the corn plants, there was reported an increase in total calcium, and an increase in ether-soluble and ether-insoluble phosphorus upon irradiation. Wynd and Fuller (18), on the basis of analyses of material from the two experiments previously reported by Fuller, claimed an increase in calcium of irradiated cucumber and tomato plants (tomato showed an increase of 0.05 to 0.06 per cent) and a decrease in phosphorus. In this paper they stated that they wished to emphasize the increase in per cent of calcium of the irradiated plants. Several years ago a large number of experiments performed in this laboratory for the purpose

of studying differences in mineral composition effected by irradiation failed to yield concordant results. Sometimes a marked increase in ash of irradiated plants occurred, and associated with this increase in ash was an increase in calcium or phosphorus, or both; at other times, irradiation failed to alter the ash content or change the calcium-phosphorus level. Later it was observed that lack of response was characteristic of irradiation during periods of high light intensity, and that increase in ash and increase in calcium or phosphorus, or both, always occurred with irradiation during periods of low light intensity. The results in Table V A furnished examples of this type of behaviour with irradiation of plants in soil under high and low light intensity. Light intensity is an important factor in determining response to irradiation; and, unless the conditions of light intensity under which plants are grown and irradiated are stated, experiments describing the effects of irradiation on plants are of little value. Although the optimum light intensity for maximum response by the plant to irradiation was not determined, the experiments with irradiation of plants grown under muslin showed that a reduction of 65 per cent of the full intensity of sunlight was sufficient to permit response under conditions of high light intensity. With sixty tests, some of which are not described in this paper, irradiation of plants grown during periods of low light intensity due to cloudiness produced an increase in ash accompanied by an increase in calcium or phosphorus, or both. The degree of cloudiness and amount of dosage varied, and so did the type and magnitude of the response, but in each instance there was a response. Magnesium and manganese were not affected by irradiation.

Nutrition of the plant also affects response by the plant to irradiation. Although the experiments with sand cultures (Table V) failed to show the effect of low-calcium nutrition conditions, because of unknown quantity of calcium in the tap water, they did indicate the effect of a nutritive medium of different composition. Plants grown in the sand were much more sensitive to injury by ultra-violet than plants in soil and gave the typical response to irradiation even under high light intensity. It would seem that the greater sensitivity of the plants in sand, indicated by the greater decrease in dry weight with irradiation, was due to the absence there of a nutritive condition effective in soil, an absence which prevented the formation of substances necessary for the protection of the plant against the penetration of injurious light. In animals the ratio of calcium to phosphorus and the level of calcium and phosphorus in the diet are important factors in determining response to irradiation. This, too, may affect response by the plant.

Shading of plants and injury by ultra-violet. Plants grown under shading cloth in sand or soil were much more sensitive to injury by irradiation than unshaded plants. Stoklasa (16) observed that plants grown in the green-

house were more easily injured by irradiation with ultra-violet than plants grown outside. Others (1) have noticed that plants grown in the dark displayed greater injury than plants in light upon irradiation. In these experiments, injury to plants grown under muslin was characterized by greater decrease in height than of dry weight, the plants outside by greater decrease in dry weight than of height. Plants grown under high light intensity were very resistant to penetration of the shorter wave lengths in the ultra-violet. The nature of this resistance as affected by thickness of cutin and cell wall, pigment formation, presence of unpigmented substances with absorption in the ultra-violet, the physical properties of variable constituents, and solarization remain to be investigated.

Interval between time of irradiation and response by the plant. The first response of plants to irradiation was an increase in ash of the leaves, and a decrease in ash of the stems. This occurred 48 to 72 hours after irradiation. Associated with the increase in ash of the leaves was an increase in calcium or phosphorus, or both. When the dosage for the single irradiation exceeded 15 seconds there was also an increase of phosphorus of the stems, the amount of the increase being progressively greater as the dosage increased from 30 to 120 seconds. It was found that the greater the dosage the shorter the interval between the time of irradiation and response by the plant, and for an equal lapse of time the greater the magnitude of the response. This early response was replaced later by an increase in ash of both leaves and stems, upon several repetitions of irradiation at varying dosage at intervals of 48 hours. Three irradiations of 20 seconds each were not enough for bringing about this change in every case, but four were ample. Four irradiations at 20 seconds, five at 10, and eight at 5 seconds brought about an increase in ash of both stems and leaves, and an increase of calcium or phosphorus, or both, of these tissues. An increase in number of irradiations accentuated the response; increase in dosage hastened it. All the plants used did not respond to irradiation; cabbage, for example, exhibited either a decrease or no change in ash, calcium, and phosphorus.

Response to irradiation and injury to the plant. Response to irradiation was independent of visible injury to the plant. The increase in ash, etc., was not proportional to the degree of injury with irradiation under the open arc. In fact plants grown outside in soil under high light intensity may be injured and show no response to irradiation. Cabbage, which failed to give the response to irradiation, with heavy dosage of ultra-violet was badly injured, yet displayed no increase in ash, calcium, or phosphorus. Irradiation through filters which removed the shorter wave lengths in the ultra-violet known to be injurious to plant cells showed the response by uninjured plants to be of the same magnitude as that of plants under the unscreened arc.

Wave lengths effective in the production of response. Using glass filters with varying transmission in the ultra-violet it was shown that the wave lengths inducing response were between 2900 and 3130 Å. This is the same region as that effective in the prevention and cure of rickets in the animal and the activation of ergosterol to form vitamin D. The same portion of the ultra-violet spectrum that causes an increase in ash, and of calcium or phosphorus or both in the animal induced a similar response in plants. Cabbage, a plant devoid of anti-rachitic properties either before or after irradiation, gave no response to irradiation through filters or irradiation under the unscreened lamp.

Effect of irradiated ergosterol. Application of olive oil saturated with irradiated ergosterol to basal leaves of plants resulted in an increase in ash even though the treated leaves were not included in the material analyzed. Experiments by the "twin-leaf" method had indicated a local effect, with response restricted to leaves receiving irradiation. Analyses of the sprayed leaves and stems of plants sprayed with olive oil containing irradiated ergosterol and with olive oil alone indicated that the response to irradiated ergosterol—increase in ash and calcium—was similar to that given by irradiation. This increase in ash and calcium due to spraying with olive oil containing irradiated ergosterol was equal in magnitude to that occurring with irradiation of the plant.

Workers who investigated the anti-rachitic properties of green plants found little or no effect with the feeding of green plants to rachitic animals (3, 7, 11, 13, 14, 19), but irradiation of the plants under a quartz mercury vapor lamp rendered them potent (3, 9, 10, 12). Alfalfa grown in Arizona and cured in the dark was found to be lacking in anti-rachitic value but the same alfalfa cured in the sun possessed anti-rachitic properties, the potency increasing with the length of exposure to the sun (15). Lojkin (12) found cabbage to be lacking in anti-rachitic value even after prolonged exposure to ultra-violet radiation. Although vitamin D may be present in plants in minute quantities, or even absent, ergosterol appears to be present in larger amounts and of much more widespread occurrence as evidenced by the anti-rachitic properties of plants after irradiation. The evidence from experiments in this paper lead us to believe that response by the plant to ultra-violet irradiation is similar to that in the animal, and that irradiation of the plant, as is the case with the animal, exerts its influence indirectly by activation of the ergosterol present in the tissues of the plant.

SUMMARY

Tomato, tobacco, *Datura*, lettuce, salvia, and cabbage plants were irradiated with varying dosage 15 inches from a quartz mercury vapor lamp and some changes in mineral composition studied.

Light intensity was found to be an important factor in determining response to irradiation. Plants grown in soil during periods of low light intensity and irradiated responded with an increase in ash accompanied by an increase in calcium or phosphorus, or both; plants grown in soil during periods of high light intensity and irradiated showed no change in ash, calcium, or phosphorus.

Irradiation of plants during periods of cloudy weather produced an increase in ash, and an increase in calcium or phosphorus, or both. This response was given in each of 60 different tests.

The reduction of light intensity (65 per cent) effected by the use of muslin as a shading cloth permitted response to irradiation by the plant during periods of high light intensity.

The first response by the plant to irradiation with a single dosage of 15, 30, 60, or 120 seconds was an increase in ash of the leaves and a decrease in ash of the stems, occurring 48 to 72 hours after irradiation. The increase in ash of the leaves was accompanied by an increase in calcium or phosphorus, or both, and there was an increase in phosphorus content of the stems if the dosage exceeded 15 seconds.

The decrease in ash of the stems characteristic of the early response to irradiation was replaced, with repeated irradiation at 48-hour intervals, by an increase in ash of both stems and leaves, and an increase of calcium or phosphorus, or both, in these tissues. Four irradiations at 20 seconds, five at 10, and eight at 5 seconds sufficed to bring about this change. A larger number of irradiations accentuated the response; greater dosage hastened it.

Nutrition of the plant affected response by the plant to irradiation. Plants grown in sand having a different mineral composition from that of the soil were much more sensitive to injury by irradiation than plants in soil, and displayed increase in ash and altered calcium-phosphorus level even with the irradiation of plants grown under high light intensity.

Manganese and magnesium contents were unaffected by irradiation.

Response to irradiation was independent of visible injury to the plant.

Plants under shading cloth in sand and soil displayed greater injury with irradiation than unshaded plants.

The wave lengths in the ultra-violet active in the production of response by the plant were the same as those effective in the prevention and cure of rickets and the activation of ergosterol—2900 to 3130 Å.

Cabbage, a plant known to be lacking in anti-rachitic properties even after irradiation, failed to show an increase in ash, calcium, or phosphorus upon irradiation.

Application of irradiated ergosterol in olive oil to the basal leaves of plants brought about an increase in ash of the plants.

Plants sprayed with irradiated ergosterol in olive oil displayed an increase of both ash and calcium content.

It is believed that the ultra-violet radiation effective in the production of an increase in ash and increase of calcium or phosphorus, or both, of irradiated plants exerts its influence indirectly by activation of the ergosterol present in the tissues of the plant.

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METABOLISM OF CITRIC, SULPHURIC, AND NITRIC ACID IN THE POTATO TUBER. AN EXPLANATION FOR THE HIGH pH OF THE JUICE OF TUBERS TREATED WITH ETHYLENE CHLORHYDRIN¹

JOHN D. GUTHRIE

When freshly-harvested potato tubers are treated with ethylene chlorhydrin and the juice expressed from two to six days after treatment, it is found that the juice of the treated tubers is more alkaline than that of the untreated controls. With favorable materials, the pH of the juice is changed from pH 6 to pH 7 by the treatments. This change has been reported in several papers from this laboratory (4, 6, 10) and has been discussed in relation to other changes that occur when potato tubers are treated with ethylene chlorhydrin. Miller (10) found that the change may be observed as early as 24 hours after the beginning of treatment. He also showed that the pH difference became slightly larger on boiling or aerating the juice and that this was due to the high carbon dioxide content of the treated juice.

The object of the present investigation was to find the explanation for the pH change. Specifically, the problem was to find by chemical analysis how the treated juice differed from the check juice. It was obvious that the treated juice was more alkaline for one or both of two reasons: (a) it contained more bases; (b) it contained less acids. Therefore the changes in the various acids and bases of the juice were investigated. Since the juice is strongly buffered, the buffer substances were also studied.

DESCRIPTION OF TREATMENTS

The treatments were made according to the dip and vapor methods of Denny (3). In the dip treatments, which were made on cut pieces, the treated sample was dipped in a solution of 40 cc. of 40 per cent of ethylene chlorhydrin per liter, the excess solution drained off, and the pieces placed in a closed container for 24 hours. The check sample received a similar treatment with water. The vapor treatments were made by placing the whole tubers in closed containers with one cc. of 40 per cent ethylene chlorhydrin per liter for 24 hours. The check tubers were kept in a closed container for 24 hours. Unless otherwise stated, the pieces treated by the dip method were planted until sampled while the whole tubers treated by the vapor method were kept in sacks in the laboratory.

Specific details of the various treatments and sources of the tubers follow:

¹ Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 74.

Treatments 1 and 2. Freshly-harvested Bliss Triumph tubers from the Institute gardens were treated by the dip method.

Treatments 3 to 8. Bliss Triumph tubers from the Institute gardens that had been in storage about three months were treated by the dip method. Treatments 4 and 6 were kept in moist chambers until sampled. Treatments 5 and 8 were planted in sand and watered with distilled water until sampled. Treatments 3 and 7 were planted in soil as usual.

Treatments 9 and 10. New potatoes of an unknown variety from the local market were used. Treatment 9 was made by the vapor method, treatment 10 was made by the dip method.

Treatments 11 and 12. New Spalding's Rose potatoes from the local market were used. Treatment 11 was made by the vapor method, treatment 12 was made by the dip method.

Treatments 13 to 16. Irish Cobbler potatoes from Charleston, South Carolina, bought in the local market, were used. Treatments 13 and 16 were made by the vapor method, treatments 14 and 15 by the dip method.

Treatments 18 to 22. Freshly-harvested potatoes from the Institute gardens were treated by the vapor method. Treatment 20 was made with Bliss Triumph tubers, treatments 18, 19, 21, and 22 with Irish Cobbler tubers.

Treatment 23. Bliss Triumph tubers from Maine were used. Three lots were treated by the vapor method and three used for checks. The juice was expressed from these separately.

Treatment 24. Bliss Triumph tubers from Maine were treated by the vapor method.

Treatment 25. Irish Cobbler tubers from Maine were used. Six lots were treated by the vapor method and six used for checks. The juice was expressed from these separately.

Treatment 26. Second crop Irish Cobbler tubers from New Jersey were used. Six lots were treated by the vapor method and six used for checks. The juice was expressed from these separately.

GENERAL METHODS

Unless otherwise stated, the juice samples were taken six days after the beginning of the treatments. The juice of each sample of the vapor treatments represents at least 8 tubers, but in most cases about 24 tubers were used. In the dip treatments about three liters of cut pieces were used. After washing and drying the samples, the callus was removed from the cut surface of the pieces treated by the dip method. In the case of the whole tubers, the potatoes were cut open to avoid the inclusion of any rotting tissue. In most of the cases the tissue was not peeled. The

tissue was then ground through the nut cutter of a food chopper, the juice squeezed out through cheesecloth, and the starch centrifuged out.

The boiled, filtered juice was prepared by pipetting the juice, usually 200 cc., into a volumetric flask, usually 250 cc., and heating this in boiling water for ten minutes. The juice was then cooled in cold water, made up to volume, and the coagulum filtered out through No. 4 Whatman paper into an Erlenmeyer flask. A layer of toluene was added, shaken with the juice, and the flask stoppered with a rubber stopper. Preserved in this way the juice could be kept for several months.

The quinhydrone electrode was used to make most of the pH measurements. In making some of the titration curves the Youden and Dobroscky (16) capillary glass electrode was used in the alkaline range. pH determinations were made on the juice after centrifuging, on the juice after aerating out the carbon dioxide with a stream of nitrogen, and on the boiled, filtered juice after aeration with nitrogen. No significant difference was noted between the latter two values.

Titration curves were made on practically all of the juices. The procedure was to place 20 cc. of the fresh juice or an equivalent amount of the boiled juice in a beaker and aerate with a stream of nitrogen for 15 minutes. Quinhydrone was then added and the titration made by successive additions of $N/10$ H_2SO_4 or $N/10$ $NaOH$, taking a pH reading after each addition. The aeration with nitrogen continued during the titration and served as an effective method of stirring. By proceeding in this way, the effect of carbon dioxide was removed from the experiments. This is important, since Miller (10) found that the treated juice contains more carbon dioxide than the check juice. Consequently the pH difference is larger after aeration of the treated and check juices. The amount of carbon dioxide varies also with the manner of obtaining the juice and the time of standing. By removing the carbon dioxide, the necessity of determining this variable factor was avoided.

ASH CONSTITUENTS

Although it seemed unlikely that the treatments should produce any large changes in the bases of the juice, it was thought best to investigate this possibility. Ash was therefore determined on the juice. The boiled, filtered juice was evaporated in a platinum dish, charred over a Bunsen burner, and ignited in a muffle furnace at about $600^{\circ}C$. The weight of ash obtained was somewhat dependent on the method of ignition. Consequently, the treated juice, and check juice were ashed simultaneously, side by side, so that each might be heated in the same way. It is for this reason that the value for the treated juice must be compared with the corresponding value for the check juice. The ash was moistened with about one cc. of concentrated sulphuric acid and again ignited. This converted the car-

bonates and oxides of the ash to sulphates and the weight of the ash was therefore increased. Table I shows that there was a very slight decrease in the ash content of the juice due to the treatments.

Calcium and magnesium should be less soluble at the more alkaline reaction of the treated juice, especially since the treated juice has been shown by Miller (10) to contain more carbon dioxide than the check juice. The more alkaline reaction and the presence of carbon dioxide should be favorable for the precipitation of calcium and magnesium carbonates. Calcium and magnesium were determined on the ash by the official methods (1, p. 104). Table I shows that the treated juice contains slightly less

TABLE I

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON THE ASH CONSTITUENTS OF THE EXPRESSED JUICE OF POTATO TUBERS

Treatment No.	Ash per 100 cc. of juice, grams		Ash as sulphate per 100 cc. of juice, grams		Magnesium, cc. N/10 per 100 cc. of juice		Calcium, cc. N/10 per 100 cc. of juice	
	Treated	Check	Treated	Check	Treated	Check	Treated	Check
13	1.011 0.965	1.037 0.988	1.312 1.408 1.221	1.375 1.435 1.267	9.0	9.6	2.2	2.5
14			1.115	1.223	7.1	8.8	2.2	4.6
15	0.941	0.955	1.273 1.206	1.325 1.268	6.5	7.9	2.8	5.1
16	1.028	1.046	1.410 1.307	1.435 1.327	7.9	9.1	1.7	3.2

calcium and magnesium than the check juice. The amount of this change is of approximately the right magnitude to account for the slight decrease in ash. The decrease in calcium and magnesium acts in the direction of a more acid juice and cannot in any way explain the high pH of the treated juice. The decrease in calcium and magnesium must, however, be taken into account in the quantitative consideration of the pH change that will be given later in the paper.

SULPHURIC ACID

Two hundred cc. of water, 20 cc. of 20 per cent hydrochloric acid, and 10 cc. of 10 per cent barium chloride were added to 50 cc. of the boiled, filtered juice. After standing overnight or longer, the BaSO_4 was separated by filtration through "ashless" paper, washed free from chlorides, and ignited in a muffle furnace. The precipitate was then digested with dilute hydrochloric acid, filtered out, and again ignited and weighed. From the weight of BaSO_4 the amount of sulphuric acid in 100 cc. of the original

juice was calculated. For convenience, this was expressed as cc. of N/10 H_2SO_4 .

The results of some of the sulphuric acid determinations are shown in Table II. All of the treatments reported in this table were dip treatments. With the exception of the 6-day sample of treatment 1, the juice was expressed from peeled tubers. The tubers used for treatments 1 and 2 were freshly harvested, while those for treatments 3 to 8 had been stored for several months. The method of storage after treatment was varied, the details of which have been given in the section on treatments. The results show that in every case the treatment decreased the sulphuric acid content

TABLE II

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON THE pH AND SULPHURIC ACID CONTENT OF THE BOILED, FILTERED JUICE OF BLISS TRIUMPH POTATOES

Treatment No.	Days after treatment	pH of boiled juice		Change in pH	Cc. N/10 H_2SO_4 to produce pH change in 100 cc.	SO_4 content of 100 cc. of juice as cc. N/10 H_2SO_4		Change in H_2SO_4 content	Fraction of pH change explained by H_2SO_4
		Treated	Check			Treated	Check		
1	6	7.02	5.97	1.05	20.0	1.5	5.6	4.1	0.26
1	11	6.86	6.10	0.76	11.0	4.2	7.4	3.2	0.29
2	10	6.63	6.09	0.54	8.5	5.7	9.1	3.4	0.40
2	14	6.53	6.10	0.43	6.0	7.4	10.2	2.8	0.47
3	7	6.75	6.15	0.60	8.5	11.5	16.3	4.8	0.56
3	9	6.86	6.41	0.45	5.6	11.5	14.8	3.3	0.58
4	8	7.17	6.44	0.73	9.6	14.1	17.3	3.2	0.33
5	7	6.80	6.32	0.48	5.3	11.2	12.3	1.1	0.21
6	7	7.08	6.41	0.67	10.0	12.7	16.5	3.8	0.38
7	7	6.75	6.41	0.34	5.0	11.9	14.5	2.6	0.52
8	9	6.76	6.34	0.42	6.0	9.8	12.7	2.9	0.48

of the juice. However, a comparison of the decrease in sulphuric acid with the amount of sulphuric acid required to produce the pH change as determined by titration shows that the decrease is too small to account for all of the pH change. The results indicate that from 20 to 50 per cent of the pH change could be explained by the decrease in sulphuric acid, but that other changes must account for the remainder.

NITRIC ACID

The nitric acid content of the juice was estimated by the method of Pucher, Vickery, and Wakeman (12). This method is based on the extraction of nitric acid with ether and avoids the interference of amide nitrogen. After extraction, the nitric acid was reduced to ammonia with Devarda's alloy and distilled into N/50 H_2SO_4 . Qualitative tests with diphenylamine were made on the juice. These were in agreement with the quantitative results. The effect of the treatments on the nitric acid content of the juice is shown in Table III. In all but one case, in which there was no change,

TABLE III

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS OF POTATO TUBERS ON THE CITRIC, SULPHURIC, AND NITRIC ACID CONTENT OF THE EXPRESSED JUICE

Treatment No.	pH of the aerated juice		Cc. of N/10 H_2SO_4 or NaOH to produce pH change	Citric acid, cc. N/10 or M/30 per 100 cc. of juice		Sulphuric acid, cc. N/10 per 100 cc. of juice		Nitric acid, cc. N/10 per 100 cc. of juice	
	Treated	Check		Treated	Check	Treated	Check	Treated	Check
10	7.23	6.04	13.0	36.6	58.5	5.2	10.8	1.7	3.3
11	6.79	6.38	7.0	38.7	50.7			0.0	1.0
12	6.78	6.44	4.7	33.5	46.3	7.6	9.2	1.8	3.2
13	7.46	6.39	12.5	34.1	53.2	6.0	8.9	0.0	0.3
14	7.62	6.30	14.0	16.6	44.7	5.6	8.6	0.9	2.1
15	7.50	6.11	17.5	17.8	40.4	1.0	4.3	0.4	1.0
16	7.33	6.16	15.0	21.4	46.5	2.0	4.7	0.5	0.5

the nitric acid content of the treated juice is lower than that of the corresponding check juice. Nitric acid is higher in the dip treatments 10, 12, 14, and 15 than in the vapor treatments 11, 13, and 16. This is due to the pieces treated by the dip method having been planted in soil for six days while the tubers treated by the vapor method were stored for the same time in paper sacks. The planted pieces took up additional nitrate from the soil. The magnitude of the decrease in nitric acid in terms of N/10 solution is not large enough to account for much of the pH change. However, with other plants which contain more nitric acid, the utilization of nitric acid in the synthesis of other nitrogen compounds might have an important influence on the pH of the juice.

HYDROCHLORIC ACID

The chloride in 20 cc. of the boiled, filtered juice of treatment 1 was precipitated with silver nitrate in the presence of nitric acid. The precipitate was dissolved in dilute ammonium hydroxide, precipitated with nitric acid, and ignited as AgCl . The juice from the treated lot had a chloride content equivalent to 15.3 cc. of N/10 HCl per 100 cc., while the corresponding value for the check lot was 15.1 cc. Since this result indicated no change in the hydrochloric acid content, this acid received no further attention.

PHOSPHORIC ACID

The phosphate in 100 cc. of the boiled, filtered juice was precipitated as magnesium ammonium phosphate, dissolved, and precipitated as ammonium phosphomolybdate. This was dissolved, precipitated as magnesium ammonium phosphate, and ignited and weighed as $\text{Mg}_2\text{P}_2\text{O}_7$. The juice of the treated lot of treatment 1 had a phosphate content equivalent to 3.0 cc. of M/10 H_3PO_4 per 100 cc. while the value for the check lot was

3.2 cc. On account of the importance attached to phosphate as a buffer of potato juice by Ingold (9) it was necessary to determine phosphoric acid on certain other juices in order to have the data as complete as possible in regard to the buffer substances of these juices. The values in cc. of $M/10 H_3PO_4$ per 100 cc. for the treated samples of treatments 13, 15, and 16 were 4.6 cc., 5.2 cc., and 4.0 cc., respectively. Corresponding values for the check juices were 4.6 cc., 5.3 cc., and 3.8 cc.

CITRIC ACID

Since changes in inorganic acids and bases were too small to account for all of the change in pH, the most likely remaining possibility lay in the organic acids. In agreement with the results of Miller (10) it was found that the treatments produced no significant change in the buffer capacity of the juice when sampled six days after treatment. It was difficult to

TABLE IV
FRACTIONATION OF THE BUFFERS OF POTATO JUICE BY PRECIPITATION

Treatment No.	Cc. N/10 to change from pH 2.7 to pH 8.0 per 20 cc. of juice					
	Boiled, filtered juice		Supernatant after $Ca(OH)_2$ precipitation		BaCl ₂ -alcohol precipitate	
	Treated	Check	Treated	Check	Treated	Check
13	28.5	28.0	22.5	20.0	15.0	18.5
15	25.3	25.0	22.5	20.0	13.2	16.4
16	28.5	27.5	21.2	18.6	14.8	16.9

reconcile this fact with any decrease in organic acids. Such a decrease should result in the treated juice being less buffered than the check juice. The possibility remained, however, that although a decrease in organic acid occurred, this was compensated by the formation of some other buffer substance. Various methods of fractionating the buffer substances were tried.

Treatment of the juice with calcium hydroxide should precipitate any citric acid in excess of the solubility of calcium citrate. Fifty cc. of the boiled, filtered juice were shaken with one g. of calcium hydroxide in a stoppered flask, allowed to stand one hour, and filtered. Five cc. of $N/1 H_2SO_4$ were added to 25 cc. of the filtrate which was aerated with nitrogen for 15 minutes and titrated with $N/10 NaOH$. Titration curves typical of such titrations on the calcium hydroxide filtrates of treated and check juice are shown in Figure 1. The amount of $N/10$ solution to change from pH 2.7 to pH 8.0 was found from the titration curves. This range was chosen, since it has been shown by Van Slyke and Palmer (15) to cover the

buffer range of the organic acids. Table IV shows that the calcium hydroxide filtrate of the treated juice is more buffered than a similar filtrate from the check juice. This is what would be expected if the treated juice contained less citric acid than the check juice and this was compensated by some other buffer substance.

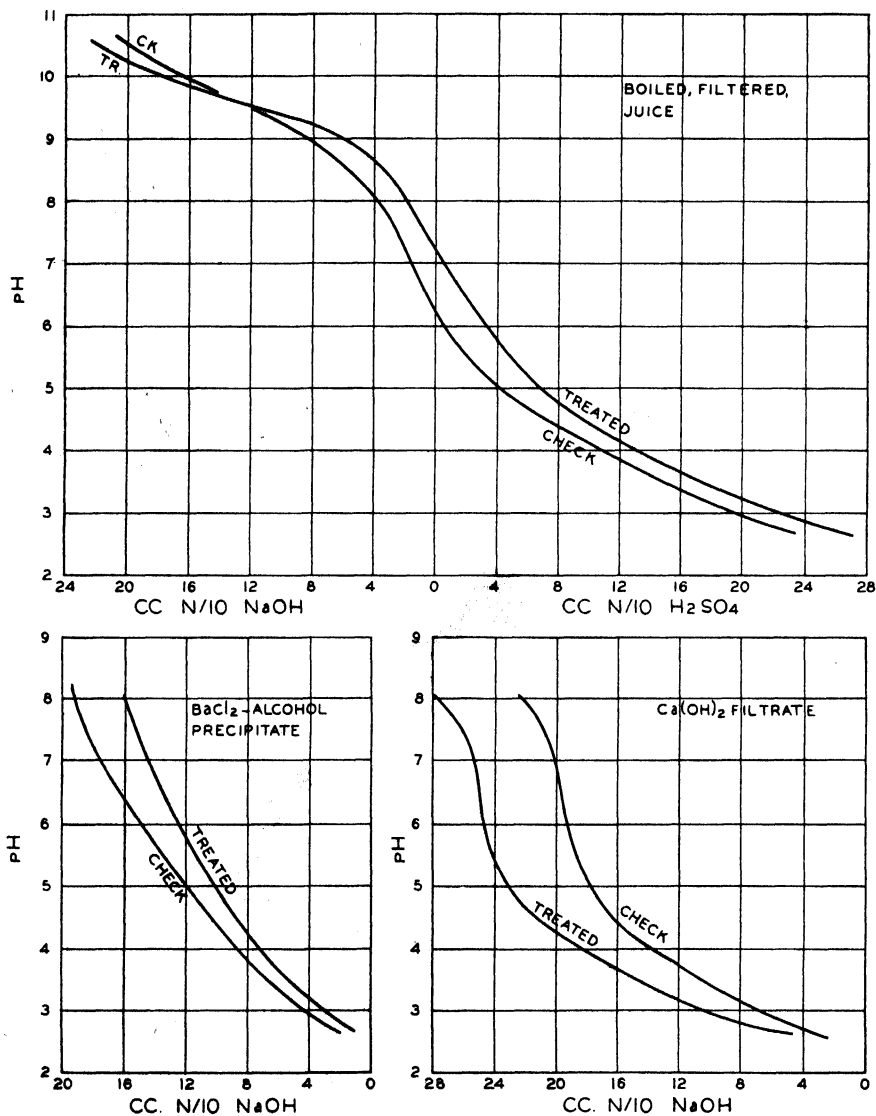


FIGURE 1. Titration curves of the boiled, filtered juice, the barium chloride-alcohol precipitate, and the calcium hydroxide filtrate of the treated and check juice of treatment 16.

Barium citrate and barium malate are insoluble in 80 per cent alcohol. The precipitate formed when BaCl_2 and alcohol were added to the boiled, filtered juice was investigated. Sufficient $\text{N}/10 \text{ H}_2\text{SO}_4$ was added to 25 cc. of the boiled, filtered juice to bring it to the pH of the check juice. An equal volume of water was added to the check juice. Ten cc. of 10 per cent barium chloride and 150 cc. of 95 per cent alcohol were added. After 1.5 hours the precipitate was separated by centrifuging and washed with 150 cc. of 80 per cent alcohol. The precipitate was taken up with 50 cc. of hot water, 3 cc. of $\text{N}/1 \text{ H}_2\text{SO}_4$ were added and allowed to stand overnight. The barium sulphate was centrifuged down and washed with 10 cc. of hot water. The supernatant liquid and washings were combined and titrated with $\text{N}/10 \text{ NaOH}$. Titration curves that are typical of the barium

TABLE V

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON THE pH AND ON THE CITRIC AND SULPHURIC ACID CONTENT OF THE EXPRESSED JUICE

Treatment No.	Days after treatment	pH of boiled juice		Cc. $\text{N}/10$ to produce pH change per 100 cc.	Cc. $\text{M}/30$ citric acid per 100 cc.		Change in citric acid $\times 0.85$	Cc. $\text{N}/10 \text{ H}_2\text{SO}_4$ per 100 cc.		Change in H_2SO_4
		Treated	Check		Treated	Check		Treated	Check	
19	3	6.96	5.98	13.5	50.0	76.3	21.5	10.6	12.8	2.2
20	3	6.42	5.85	10.5	62.7	81.7	16.2	6.0	6.7	0.7
21	3	6.85	5.97	13.5	61.6	84.1	19.1	11.1	11.5	0.4
21	6	6.82	6.04	12.5	55.3	82.0	22.7	10.6	12.6	2.0
22	3	6.62	6.09	9.0	59.0	75.6	14.1	10.5	12.6	2.1
22	6	6.59	6.09	8.0	59.4	77.0	15.3	11.6	12.6	1.0

chloride-alcohol precipitates of treated and check juice are shown in Figure 1. The values listed in Table IV show that the barium chloride-alcohol precipitate of the treated juice requires less $\text{N}/10$ solution to change it from pH 2.7 to pH 8.0 than the corresponding precipitate from the check juice. Similar results were obtained by the use of barium nitrate or calcium chloride in place of the barium chloride. These results were interpreted as indicating a decrease in some organic acid precipitated by barium chloride and alcohol. Since the calcium hydroxide procedure indicated that this was citric acid, this possibility was investigated.

Citric acid was determined by the official pentabromacetone method of Hartmann and Hillig (8), using 50 cc. of the boiled, filtered juice. The results of these determinations are shown in Tables III and V. In all cases the treated juice contains less citric acid than the check juice. Table V shows that the change in citric acid is as large on the third day after treatment as on the sixth day. This is also true of the amount of $\text{N}/10$ solution to produce the pH change. The change in citric acid is rather large, averaging 20.8 cc. of $\text{N}/10$ citric acid per 100 cc. of juice. A comparison of this

value with the amount of N/10 solution necessary to change the treated juice to that of the check indicates that the change in citric acid together with the change in sulphuric and nitric acid is more than enough to account for the pH change. This point will receive further attention in the next section.

COMPARISON OF THE OBSERVED CHANGE IN ACIDITY WITH THE ANALYTICAL RESULTS

In order to see how nearly the changes in acids and bases that have been listed in the previous sections explain the pH change, a balance sheet has been prepared in which the amount of acid or base required to pro-

TABLE VI

BALANCE SHEET COMPARING THE CHANGE IN ACID BASE CONTENT OBTAINED FROM TITRATION CURVES WITH THE VALUES OBTAINED BY ANALYSIS OF THE JUICE. ALL VALUES ARE CC. N/10 SOLUTION PER 100 CC. OF JUICE

	Treatment No. 13	Treatment No. 14	Treatment No. 15	Treatment No. 16
Cc. N/10 to produce pH change	12.5	14.0	17.5	15.0
Decrease in citric acid $\times 0.85$	16.2	23.9	19.2	21.3
Decrease in sulphuric acid	2.9	3.0	3.3	2.7
Decrease in nitric acid	0.3	1.2	0.6	0.0
Total decrease in acids	19.4	28.1	23.1	24.0
Decrease in magnesium	0.6	1.7	1.4	1.2
Decrease in calcium	0.3	2.4	2.3	1.5
Total decrease in bases	0.9	4.1	3.7	2.7
Net decrease in acidity	18.5	24.0	19.4	21.3

duce the pH change per 100 cc. of juice as determined by titration is compared with the change in acids and bases obtained from the analytical results. In order to do this, it was necessary to express all values in the same terms. Since citric acid is less effective in changing the pH of the buffer than sulphuric acid, a factor was determined for converting the citric acid change into terms of sulphuric acid. This was done by finding the amount of sulphuric acid required to change a given amount of potato juice from pH 7 to pH 6 and comparing this value with the amount of citric acid to produce the same change. The factor for converting M/30 or N/10 citric acid into terms of sulphuric acid was found to be 0.85.

Table VI shows the comparison of the amount of acid or base required to produce the pH change with the changes in acids and bases in the four experiments in which the data are most complete. The net decrease in acidity, calculated from the analytical results, is consistently larger than expected from the titration values. This is also found to be true when the

analytical values for other juices on which the data are not so complete are examined, as for example Table V. This may possibly be due to a constant error in the citric acid determinations, leading to a high value for the citric acid decrease, but is more likely due to the decrease in citric acid being partially compensated by a small increase in some other acid, such as malic acid. Table IV shows that the change in buffer value of the barium chloride-alcohol precipitate is slightly smaller than would be expected from the citric acid change observed in the same treatments. Since this precipitate is composed largely of barium citrate and barium malate, this result is consistent with a small increase in malic acid. A more definite answer must await the development of a convenient method for malic acid.

Oxalic acid was estimated in the boiled, filtered juices of treatments 13 and 15 by acidifying the juice with acetic acid, removing the slight coagulum and adding calcium chloride. After standing overnight the precipitate was washed thoroughly with water by centrifuging, dissolved in warm, dilute sulphuric acid, and titrated with $N/20$ $KMnO_4$. This procedure probably gives maximal values. The treated samples gave values of 4.1 cc. and 3.2 cc. of $N/10$ oxalic acid per 100 cc., while the corresponding values for the check juices were 3.6 cc. and 2.2 cc. These results, even if taken at their face value, cannot account for the discrepancy discussed above.

If the ash after ignition with sulphuric acid is considered as composed chiefly of the sulphates of potassium, calcium, and magnesium, it is possible to calculate the equivalents of base in the juice. In these calculations the magnesium and calcium data in Table I were used, and the remaining ash calculated as potassium sulphate. The values for the treated samples of treatments 13, 15, and 16 are 15.4, 14.6, and 15.9 milliequivalents per 100 cc. of juice, while the corresponding values for the check juices are 16.0, 15.2, and 16.2, milliequivalents. In order to see how nearly the acids which were investigated account for the bases, calculations, based on the barium chloride-alcohol precipitate, were made. This precipitate includes citric, malic, oxalic, and phosphoric acid. The barium chloride-alcohol precipitate values were multiplied by 5, to put them on the basis of 100 cc. of juice, and divided by 0.91, since Van Slyke and Palmer (15) have shown that it takes 9.1 cc. of $N/10$ $NaOH$ to titrate 10 cc. of $N/10$ citric acid from pH 2.7 to pH 8.0. The sulphuric acid values and 1.5 milliequivalents to represent the approximate hydrochloric acid were added to this. The values for the treated samples of treatments 13, 15, and 16 are 10.4, 8.8, and 10.0 milliequivalents, while the values for the corresponding check juices were 12.9, 10.8, and 11.5 milliequivalents. This shows that approximately three-fourths of the acids in the juice are accounted for by sulphuric acid, hydrochloric acid, and the acids precipitated by barium

chloride and alcohol. The treated juices have more bases unaccounted for by acids than the check juices. This would be expected from their higher pH values.

BUFFER CAPACITY OF THE JUICE

During the course of the investigation numerous titration curves were made on treated and check juice. In agreement with the results of Miller

TABLE VII

INFLUENCE OF THE TIME OF SAMPLING ON THE EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON THE BUFFER CAPACITY OF THE EXPRESSED JUICE

Treatment No.	Cc. N/10 to change 20 cc. of juice from pH 8.0 to pH 2.7							
	3 days after treatment				6 days after treatment			
	Fresh juice		Boiled juice		Fresh juice		Boiled juice	
	Treated	Check	Treated	Check	Treated	Check	Treated	Check
18	32.0	35.5						
19	30.0	34.2	28.5	32.0				
20	33.0	36.0	30.3	32.6				
21	35.0	37.0	32.5	34.0	35.0	35.3	33.0	33.5
22	35.5	37.0	31.7	33.2	36.0	35.5	33.1	33.4
23	35.5	36.4	32.8	34.2	36.5	36.8	33.2	33.2
	36.0	36.0	33.2	33.5	37.4	37.9	33.7	33.5
	35.0	38.1	32.0	35.5	37.5	36.8	34.0	33.2
24	36.1	39.5	33.2	36.0	39.3	39.5	37.2	37.5
25	43.5	42.9	38.5	37.0	46.5	44.1	39.5	38.5
	38.5	43.5	36.1	37.1	42.0	43.2	37.5	34.0
	42.2	41.0	36.5	36.5	43.0	42.6	38.7	38.0
	41.0	36.1	40.8	35.0	38.5	42.5	35.5	39.0
	41.0	37.2	35.5	35.5	41.3	42.5	38.5	39.0
	40.3	41.3	35.5	36.5	40.0	41.7	37.0	38.0
26	36.4	37.4	33.4	35.5	36.7	37.0	35.0	35.0
	37.0	38.0	35.0	35.6	37.0	38.0	35.0	35.7
	35.2	38.8	33.5	36.5	37.0	37.0	34.8	35.8
	35.7	38.2	32.5	35.1	37.0	36.2	37.4	35.6
	36.5	37.5	33.9	34.4	36.8	38.5	36.2	37.0
	34.8	37.7	31.7	35.0	37.7	37.6	37.1	36.6

(10) it was found that no significant change could be shown in the buffer capacity of the juice when the samples were taken six days after treatment. Since citric acid was found to be lower in the treated juice, it was expected that the treated juice should have less buffer capacity than the check juice, unless the lost buffer capacity due to the decrease in citric acid was compensated by the development of another buffer substance. Most of the treatments were sampled six days after treatment, but a few sampled at an earlier date showed a slightly lower buffer value for the treated juice. The results of an investigation of this point are given in Table VII. With the exception of treatment 25, all the three-day samples

show a lower buffer value for the treated juice. This is true of both the fresh and the boiled, filtered juice. The explanation for the failure to get this result with treatment 25 lies in the extreme variability of the tubers used for this treatment with regard to the buffer capacity of the juice. The average error for the buffer value of the juice of the 12 check lots of this treatment is ± 1.8 cc., while the average error for the 12 check lots of treatment 26 is ± 0.5 cc., and the average error for the 6 check lots of treatment 23 is ± 0.7 cc. In agreement with previous results, no significant difference can be shown in the buffer capacity of the juices expressed six days after treatment. These results are interpreted to mean that the buffer substance which compensates for the buffer action of the citric acid develops at some time between the third and sixth day after treatment.

There are reasons for thinking that this compensating buffer is an amphoteric substance. If it were another organic acid, it would replace not only the buffer capacity of the citric acid but its acidity as well and there would be a much smaller pH change. Furthermore, the formation of such an acid buffer would make the pH difference become less on the sixth day. This is not the case. Since practically all known amphoteric compounds in plants are nitrogenous substances and most of them amino compounds, the total nitrogen, amino nitrogen, and amide nitrogen of the juice were investigated.

NITROGEN COMPOUNDS

Total nitrogen. It was observed that during prolonged heating of potato juice on the steam bath the juice became more acid. This was interpreted as being due to a slow hydrolysis of amides, accompanied by a loss of ammonia and the formation of aspartic or glutamic acid. It was observed that the steam distillate of potato juice contained ammonia. Such a loss of ammonia during prolonged heating of potato juice should result in a slight loss in total nitrogen. It should be possible to avoid this by adding sulphuric acid before evaporating the juice and thus retain all the ammonia. In one experiment 25 cc. of boiled juice and 50 cc. of water were evaporated to dryness and the total nitrogen compared with that obtained from a similar experiment to which 3 cc. of concentrated sulphuric acid were added. The addition of 0.2 g. of calcium carbonate was also tried. The total nitrogen for the average of duplicates was 47.3 mg. without added substance, 47.8 mg. with 3 cc. of sulphuric acid, and 46.1 mg. with 0.2 g. of calcium carbonate. A similar experiment gave the values 48.3, 49.6, and 47.0 mg., respectively. As a further check on this point, six determinations were made without and six with sulphuric acid. The total nitrogen without sulphuric acid was 48.1 mg. with an average error of ± 0.3 mg., while with sulphuric acid the value was 49.6 mg. with an average error of ± 0.2 mg.

The procedure finally adopted for total nitrogen was to place 20 cc. of the boiled, filtered juice in a Kjeldahl flask, add 5 cc. of concentrated sulphuric acid, 2 g. of iron powder, evaporate almost to dryness, add 30 cc. concentrated sulphuric acid, a crystal of copper sulphate, and digest until almost colorless. Ten grams of anhydrous sodium sulphate were then added and the digestion continued at least one hour after the solution became colorless.

TABLE VIII

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON THE TOTAL NITROGEN AND AMINO NITROGEN CONTENT OF THE EXPRESSED JUICE OF POTATO TUBERS

Treatment No.	Total N, mg. per 100 cc.		Amino N, mg. per 100 cc.		Non-amino N, mg. per 100 cc.	
	Treated	Check	Treated	Check	Treated	Check
9	316	309	165	152	149	157
10	314	287	168	149	146	138
11	303	278	156	143	147	135
12	304	292	163	141	141	151
13	205	189	120	105	85	84
14	190	165	108	92	82	73
15	195	188	114	106	81	82
16	242	210	144	128	98	82
17	185	175	107	102	78	73

TABLE IX

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS OF POTATO TUBERS ON THE NITROGEN COMPOUNDS OF THE EXPRESSED JUICE

Treatment No.	Total N, mg. per 100 cc.				Amino N, mg. per 100 cc.				Amide N, mg. per 100 cc. 6 days after treatment	
	Days after treatment				Days after treatment					
	3		6		3		6			
	Tr.	Ck.	Tr.	Ck.	Tr.	Ck.	Tr.	Ck.	Tr.	Ck.
21	254	245	278	251	124	120	144	116	57	57
22	247	249	272	251	126	122	141	126	57	57
23	240	238	255	231	116	112	133	117	44	45
24	252	268	280	264	120	127	149	124	51	50
26	216	218	242	212	109	104	128	103	43	45
26	213	208	244	210	102	103	156	108	45	46

The total nitrogen results are given in Tables VIII and IX. In every case the treatment produced a small increase in the total nitrogen content of the expressed juice when the tubers were sampled six days after treatment. No change had occurred three days after treatment. This would be expected if the buffer that compensates for the citric acid is a nitrogen compound.

Amino nitrogen. Since most of the amphoteric buffer substances in

plants are amino compounds, amino nitrogen was determined on the boiled, filtered juice by the Van Slyke method (14). Since experiments had shown that the ammonia content of the juice was negligible, the determinations were made directly on the boiled, filtered juice after appropriate dilution. The results are given in Tables VIII and IX. An increase in amino nitrogen is found six days after treatment, but no increase is apparent three days after treatment. The amino nitrogen accounts for the total nitrogen increase. These results are in agreement with the supposition that the compensating buffer substance is an amino compound.

The buffer curves of some of the amino acids and dipeptides may be found in a paper by Eckweiler, Noyes, and Falk (5). An examination of these shows that the buffer action of simple amino acids, such as glycine, alanine, or leucine, is not large enough in the range covered by citric acid to compensate quantitatively for the decrease in citric acid observed in the experiments. The buffer action of the dipeptides, such as glycyl-glycine, alanyl-glycine, and alanyl-alanine, have sufficient buffer action in the range covered by citric acid to compensate for the decrease in citric acid. The formation of a dipeptide, however, would call for an increase in total nitrogen twice that of amino nitrogen. The data show that the increase in total nitrogen is approximately equal to the increase in amino nitrogen. Although it is possible to postulate mechanisms by which a dipeptide could be formed while the increase in total nitrogen was equal to the increase in amino nitrogen, the analytical results must be considered unfavorable to the dipeptide explanation.

Since glutathione is increased by the treatments (7) this slightly complicates the nitrogen situation. The glutathione increase amounts to about 30 mg. per 100 cc. with favorable treatments. This is equivalent to 4 mg. of nitrogen and is not a very large part of the total nitrogen increase which averages 20 mg. per 100 cc. of juice.

Amide nitrogen. Twenty cc. of boiled, filtered juice were diluted with 20 cc. of water and refluxed with 2.5 cc. of concentrated sulphuric acid for 2.5 hours. This was cooled, made up to 100 cc., and a 25 cc. aliquot placed in an aeration tube. Five drops of capryl alcohol, 2.2 cc. of 20 per cent NaOH, and 20 cc. of 52 per cent potassium carbonate were added, the ammonia aerated into 25 cc. of N/50 H_2SO_4 for two hours, and the excess acid titrated with N/50 sodium hydroxide.

The results are shown in Table IX. The amide nitrogen is not significantly changed by the treatments. This shows that the amino compound that is increased by the treatments is not asparagine or glutamine. For the sake of completeness, amide nitrogen was determined on treatments 13, 15, and 16. The values for the treated lots were 29 mg., 29 mg., and 36 mg., respectively, while the values for the corresponding check lots were 29 mg., 30 mg., and 34 mg.

BUFFERS OF POTATO JUICE

During the course of the investigation which was directed primarily to finding the changes in the expressed juice that bring about the pH change, considerable data accumulated which have a bearing on the problem of the nature of the buffer substances present in potato juice. Numerous titration curves were made and in some of them the titrations were carried to pH 10 by means of the Youden and Dobrosky (16) capillary glass electrode. A typical curve for treated and check juice is shown in Figure 1. The juice was sampled six days after treatment. At this stage there is no significant change in the buffer capacity in the acid range, but the treated juice is more buffered in the alkaline range. In order to show

TABLE X

SUMMARY OF BUFFER VALUES. VALUES ARE CC. N/10 SOLUTION TO CHANGE 20 CC. OF JUICE FROM PH 7 TO PH 10 AS DETERMINED WITH THE GLASS ELECTRODE 6 DAYS AFTER BEGINNING OF TREATMENT

Treatment No.	Treated	Check	Difference
13	15.7	14.0	+1.7
15	15.6	15.8	-0.2
16	18.0	14.5	+3.5
21	18.8	18.6	+0.2
22	20.4	18.8	+1.6
23	17.3	15.3	+2.0
	18.2	16.4	+1.8
	17.2	15.6	+1.6
24	21.0	18.5	+2.5
Av. difference			+1.6

this more conclusively, the amount of N/10 sodium hydroxide necessary to change the juice from pH 7 to pH 10 has been listed for several treatments in Table X. In all but one case the treatments increased this value. This is what one would expect from the amino nitrogen increase, since amino acids are good buffers in this range.

In order to see how much of the buffer capacity of the juice in the alkaline range is due to amino compounds, the buffer indices of the boiled, filtered juice were compared with the buffer indices of the asparagine content calculated from the amide nitrogen and with the buffer index of the other amino compounds calculated as glycine. The buffer values for glycine were taken from the paper of Eckweiler, Noyes, and Falk (5), while the values for asparagine were calculated from a titration of M/10 asparagine with the glass electrode. The results, given in Table XI, show that all of the buffer action of the check juice in the range of pH 8 to pH 10 and almost all of the buffer action of the treated juice in this range

may be accounted for by amino compounds. The discrepancy in the case of the treated juice is probably due to the presence of an amino compound having a different buffer capacity than a simple amino acid.

The buffer indices from pH 3 to pH 8 of the boiled, filtered juice and various fractions of the same juice are given in Table XII. Barium chloride and alcohol precipitate a large part of the buffer substances. This precipitate includes citric and malic acid. It also includes phosphoric acid and any oxalic acid that might be present. In the range from pH 5 to pH 4 about half of the buffer action of the barium chloride-alcohol precipitate of the treated juice is due to citric acid while somewhat more than half

TABLE XI

COMPARISON OF THE BUFFER ACTION OF THE BOILED JUICE OF POTATO TUBERS IN THE ALKALINE RANGE WITH THE BUFFER ACTION CALCULATED FROM THE CONTENT OF ASPARAGINE AND AMINO ACIDS

	Treat- ment No.	Buffer index $\times 1000$			
		pH 10 to 9		pH 9 to 8	
		Tr.	Ck.	Tr.	Ck.
Boiled juice	13	45	36	22	24
Due to asparagine		6	6	11	11
Due to other amino compounds		35	29	9	8
Boiled juice	15	47	43	20	22
Due to asparagine		6	6	11	11
Due to other amino compounds		33	29	9	8
Boiled juice	16	60	40	20	23
Due to asparagine		7	7	13	13
Due to other amino compounds		30	36	10	9

of the buffer action of the barium chloride-alcohol precipitate of the check juice is due to citric acid. Since malic acid buffers well in this range, the maximal value for malic acid is approximately that of the citric acid. This agrees with the results of Ingold as reported by Small (13, p. 291) who finds the malic acid content of potato juice approximately that of citric acid. However, the values for citric acid found in these experiments range from about 0.013 molar to 0.027 molar, which are much higher than the value 0.0057 molar given by Ingold. The buffer indices for the boiled, filtered juice are also higher than the values given by Ingold. This point has also been observed by Millér (10) who found higher buffer indices than Ingold.

Since the buffer action of malic acid is negligible in the ranges pH 8 to pH 7 and pH 7 to pH 6 (13, p. 358), it would be expected that in these ranges the sum of the buffer indices of the phosphoric acid and the citric acid should be approximately that of the barium chloride-alcohol precipi-

tate. This is found not to be the case and indicates that barium chloride and alcohol precipitate some other substance effective in this range. Amino nitrogen determinations on the barium chloride-alcohol precipitate show that it contains very little amino nitrogen, the amount found being 8 mg. per 100 cc. of juice. The unknown buffer substance carried down in this range by barium chloride and alcohol is probably not an amino compound.

TABLE XII

COMPARISON OF THE BUFFER ACTION OF THE BOILED JUICE OF POTATO TUBERS WITH THE BUFFER ACTION OF VARIOUS COMPONENTS AND FRACTIONS OF THE SAME JUICE

	Treatment No.	Buffer index $\times 1000$									
		pH 8 to 7		pH 7 to 6		pH 6 to 5		pH 5 to 4		pH 4 to 3	
		Tr.	Ck.	Tr.	Ck.	Tr.	Ck.	Tr.	Ck.	Tr.	Ck.
Boiled juice	13	12	9	13	13	16	18	37	36	43	44
BaCl ₂ -alcohol precipitate		5	8	9	12	12	17	18	23	23	24
Ca(OH) ₂ filtrate		6	5	6	4	12	9	30	25	41	40
Due to citric acid		0	0	2	4	7	11	9	13	8	11
Due to asparagine		2	2	0	0	0	0	0	0	2	2
Due to other amino compounds		0	0	0	0	0	0	0	0	13	11
Due to phosphoric acid		2	2	2	2	0	0	0	0	0	0
Boiled juice	15	13	12	12	11	16	15	29	29	40	39
BaCl ₂ -alcohol precipitate		6	8	9	13	11	15	12	17	19	20
Ca(OH) ₂ filtrate		7	10	6	3	9	7	24	23	44	38
Due to citric acid		0	0	2	3	4	9	6	11	5	9
Due to asparagine		2	2	0	0	0	0	0	0	2	2
Due to other amino compounds		0	0	0	0	0	0	0	0	12	11
Due to phosphoric acid		2	2	2	2	0	0	0	0	0	0
Boiled juice	16	12	12	15	12	16	17	32	32	45	48
BaCl ₂ -alcohol precipitate		7	10	11	12	11	15	13	16	21	21
Ca(OH) ₂ filtrate		11	10	4	3	7	7	24	19	41	37
Due to citric acid		0	0	2	4	5	10	6	13	5	11
Pb(NO ₃) ₂ -alcohol precipitate		6	7	10	11	15	14	17	21	21	22
Hg(NO ₃) ₂ after lead nitrate		6	7	3	2	2	2	3	4	13	14
NaOH after Hg(NO ₃) ₂ and Pb(NO ₃) ₂		3	3	1	1	3	3	10	10	16	15
Due to asparagine		3	3	0	0	0	0	0	0	3	3
Due to other amino compounds		0	0	0	0	0	0	0	0	14	13
Due to phosphoric acid		2	2	2	2	0	0	0	0	0	0

The filtrate from the calcium hydroxide precipitation is slightly more buffered for the treated juice. This is expected from the lower citric acid content of the treated juice, since citric acid is precipitated by the addition of calcium hydroxide. Phosphoric acid should also be removed by this treatment. The sum of the buffer indices of the calcium hydroxide filtrate added to the buffer indices of the citric and phosphoric acid might be expected to approximate the buffer indices of the boiled, filtered juice. This is found to be the case except for the range of pH 7 to pH 6. This

indicates that calcium hydroxide precipitates some other substance that has buffer action in this range.

Amino acids and asparagine are an important part of the buffer system between pH 4 and pH 3. The substances of the barium chloride-alcohol precipitate and the amino compounds account for most of the buffer action for pH 4 to pH 3.

The lead nitrate-alcohol precipitate has about the same buffer action as the barium chloride-alcohol precipitate. It is also low in amino nitrogen. Mercuric nitrate was added to the supernatant liquid from this precipitation. The amino nitrogen content of the mercuric nitrate precipitate was 84 mg. per 100 cc. of juice for the treated sample and 79 mg. per 100 cc. of juice for the check sample. The buffer indices of this fraction show a high value in the range of pH 4 to pH 3, which is in agreement with its high amino nitrogen content. The supernatant liquid from this precipitation was neutralized with $N/1$ NaOH. The precipitate that formed contained amino nitrogen equivalent to 38 mg. per 100 cc. of treated juice and 29 mg. per 100 cc. of check juice. The buffer indices of this fraction show that it contains buffer substances in addition to the simple amino acids. The results also show that lead nitrate, followed by mercuric nitrate, followed by sodium hydroxide, precipitates substantially all of the buffer substances of the juice. Hydrogen sulphide was used in decomposing the mercury and lead precipitates.

It may be concluded from the above results and discussion, that a large part of the buffer action of potato juice is accounted for by citric acid, malic acid, amino acids, and asparagine. Phosphoric acid is responsible for a small part of the buffer action between pH 8 and pH 6.

DISCUSSION

The sulphuric acid decrease is of interest in relation to the increase in glutathione in the tubers. The possibility that the sulphuric acid is converted into glutathione has been suggested in a previous paper (7). The average decrease in sulphuric acid, calculated from the data, is 3.1 cc. $N/10$ per 100 cc. of juice. This would supply sulphur for 48 mg. of glutathione. The increase in glutathione ranges from 10 to 30 mg. per 100 g. of tissue. It appears that the decrease in sulphuric acid is larger than would be necessary to explain the increase in glutathione. It may be that other sulphur compounds are involved or that the glutathione increase has been underestimated. A more thorough investigation of the relation between the decrease in sulphuric acid and the increase in glutathione is planned.

The decrease in nitric acid may be connected in some way with the increase in amino nitrogen, although quantitatively the increase in amino nitrogen is much greater than the decrease in nitrate nitrogen. Furthermore, the increase in amino nitrogen takes place in tubers in which nitric

acid is very low or absent. However, it would be of interest to study the nitrogen changes in tubers of a high nitrate content or tubers whose nitrate content had been artificially increased.

It is probable that the decrease in citric acid is due to utilization of this acid in respiration, since Miller (11) has shown that the respiration of treated tubers is much higher than that of check tubers. Such a connection would indicate that citric acid may be an intermediate compound in the breakdown of starch and sugars to carbon dioxide. In theoretical discussions of the respiratory process in plants a rôle is frequently given to malic acid. For a summary of such hypotheses the reader is referred to the paper of Bennet-Clark (2). The results show that in the potato tuber it is citric acid that undergoes rapid changes under conditions of high respiration. Citric acid might well receive more attention in plant physiological investigations.

The explanation of the buffer action of potato juice is still far from complete. Important buffer substances which have not been identified are present in the juice. This is especially true of the range of pH 8 to pH 6, which includes the natural pH of the juice and is probably important in the physiology of the tuber. The exact nature of the amino compounds also remains to be determined, especially the compound which compensates for the loss in buffer action due to the lower citric acid content of the treated juice.

The results of the experiments show that it is possible to explain the pH of a plant juice on the basis of the chemical compounds contained in it and that changes in the pH of the juice may be explained by metabolic changes which increase or decrease the quantity of these compounds. The pH of the juice is not necessarily a factor controlling the metabolic processes of the plant but can be regarded as an end result of these processes.

SUMMARY

1. Ethylene chlorhydrin treatments of dormant potato tubers result in a decrease in the citric, sulphuric, and nitric acid content of the expressed juice. These decreases in terms of cc. N/10 solution per 100 cc. of juice average 20.8 cc., 3.1 cc., and 1.0 cc., respectively.

2. The juice of the treated tubers is slightly lower in ash than the check juice. This is due to a small decrease in calcium and magnesium.

3. The net result of these changes in acids and bases is of the right order of magnitude to account for the observed increase in pH which results from the treatments. The net change is, however, consistently larger than would be required to explain the pH increase. This may be due to some constant error in the methods or to some substance that was not determined, such as malic acid.

4. The treated juice is slightly less buffered in the acid range when the

samples are taken three days after treatment. The difference is not observed when the samples are taken six days after treatment. The difference on the third day is interpreted as being due to the citric acid decrease, while the disappearance of the difference on the sixth day is interpreted as being due to the development of an amphoteric substance that compensates for the buffer action of the lost citric acid.

5. There is no change in the total nitrogen or amino nitrogen content of the expressed juice on the third day after treatment, but on the sixth day the treated juice contains more total nitrogen and more amino nitrogen than the check juice. No change was found in amide nitrogen.

6. Unless potato juice is acidified with sulphuric acid prior to evaporation for total nitrogen determinations, a slight loss in nitrogen may occur.

7. The buffers of potato juice were fractionated by various methods. Amino acids and asparagine account for the larger part of the buffer action of the juice from pH 8 to pH 10. Citric acid, malic acid, amino acids, and asparagine account for the larger part of the buffer action of the juice from pH 8 to pH 3, but important buffer substances are present, the nature of which is unknown.

8. Phosphoric acid is responsible for a small part of the buffer action of the juice between pH 8 and pH 6.

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ORGANIC THIOCYANOGEN COMPOUNDS AS INSECTICIDES¹

ALBERT HARTZELL AND FRANK WILCOXON

Comparatively few organic thiocyanogen compounds are mentioned in the literature as having been tested for their insecticidal properties. Moore (7) in an investigation of the toxicity of the vapors of a large number of organic compounds to houseflies (*Musca domestica* L.) showed that allyl isothiocyanate was highly toxic. This substance was also found by Tattersfield and Roberts (13) to be more toxic to wireworms (*Agriotes* sp.) than any other tested. In a study of more than one hundred compounds as possible fumigants against the rice weevil (*Sitophilus oryza* L.), the flour beetle (*Tribolium confusum* Farb.), and the granary weevil (*Sitophilus granarius* L.), Neifert and coworkers (10) found methyl and ethyl thiocyanate and allyl isothiocyanate more toxic than carbon disulphide.

Roark and Cotton (11) found iso-propyl thiocyanate, as well as ethyl and allyl isothiocyanate, highly effective as fumigants against the rice weevil. More recently Murphy and Peet (8, 9) have presented data showing the high toxicity of an aliphatic thiocyanate to *Aphis rumicis* L. and to the citrus mealy bug (*Pseudococcus citri* Risso) when used as a spray material. These authors, however, neglected to mention the name of the thiocyanate used. Drake and Busbey (1) studied the comparative toxicity of several thiocyanates and isothiocyanates to goldfish. There are also a considerable number of references in the patent literature to the use of thiocyanogen compounds as insecticides (5, 14, 16, 17). In view of the numerous indications that certain compounds containing the SCN group show high toxicity to insects, it was decided to prepare and test a number of such compounds, representing various types.

METHODS OF PREPARING THIOCYANOGEN COMPOUNDS

There are several methods available for the introduction of the SCN group into organic compounds. Many halogen-substituted compounds react readily with potassium thiocyanate, yielding the corresponding thiocyanogen compound and potassium halide (18, 19, 20). The ease with which this reaction takes place varies with the particular compound used, and bromine-substituted compounds react more readily than those containing chlorine.

In the case of compounds containing an aromatic nucleus, direct substitution using a solution of free thiocyanogen takes place in certain cases (12), or the thiocyanogen may be generated by the action of bromine on

¹ Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 75.

sodium thiocyanate in a solvent such as glacial acetic acid (6). Finally the diazotization of an aromatic amine, with addition of potassium thiocyanate and cuprous thiocyanate (2), leads to the introduction of the SCN group into the ring.

Phenyl thiocyanate. This was prepared from aniline, as described by Gattermann and Haussknecht (2) making use of the Sandmeyer reaction. Sulphur was determined by oxidation with potassium perchlorate and sodium peroxide in the Parr bomb. *Analysis.* Calc. for C_7H_5NS : S, 23.72. Found: S, 23.6.

N-butyl thiocyanate and methyl thiocyanate. These were obtained from the Eastman Kodak Company and were used without further purification.

Methyl thiocyanacetate. Methyl chloracetate (Eastman) was refluxed in alcohol with an equivalent of potassium thiocyanate. The product boiled at 125° to $126^\circ C.$ at 24 mm. *Analysis.* Calc. for $C_4H_5NO_2S$: S, 24.45. Found: S, 24.0.

Ethyl α - and β -thiocyanopropionates. The corresponding bromopropionates (Eastman) were refluxed in alcohol with potassium thiocyanate. The α -compound distilled at 120° to 122° at 25 mm. while the β -compound distilled at 130° to 132° at 23 mm. *Analysis.* Calc. for $C_6H_9NO_2S$: S, 20.14. Found: S, 20.5, 20.4.

γ -thiocyanopropyl phenyl ether. From γ -bromopropyl phenyl ether (Eastman) and potassium thiocyanate in alcohol. The product distilled at 188° to 191° at 14 mm. *Analysis.* Calc. for $C_{10}H_{11}NOS$: S, 16.59. Found: S, 16.5.

β -thiocyanoethyl phenyl ether. From β -bromoethyl phenyl ether (Eastman) and potassium thiocyanate in alcohol. The substance melted at 21.1° (uncorr.). *Analysis.* Calc. for C_9H_9NOS : S, 17.89. Found: S, 18.13.

β -thiocyanoethyl ethyl ether. From β -bromoethyl ethyl ether (Eastman) and KSCN in alcohol. The product boiled at 95° to 96° at 23 mm. *Analysis.* Calc. for C_6H_9NOS : S, 24.44. Found: S, 24.3.

β -thiocyanoethyl methyl ether. β -bromoethyl methyl ether was obtained by treating ethylene glycol monomethyl ether with phosphorus tribromide in the presence of pyridine. The product was refluxed with KSCN in alcohol. The thiocyanogen compound distilled at 92° to 93° at 25 mm. *Analysis.* Calc. for C_4H_7NOS : S, 27.37. Found: S, 27.00.

2-thiocyano-octane. From 2-bromooctane (Eastman) and KSCN. The product distilled at 123° to 126° at 20 mm. *Analysis.* Calc. for $C_8H_{17}NS$: S, 18.73. Found: S, 18.6.

p-thiocyanoaniline. This compound was prepared by the method of Kaufmann and Oehring (6) by treating aniline in 96 per cent acetic acid to which sodium thiocyanate had been added with bromine in acetic acid. The product after recrystallization from hot water melted at 56.5° (uncorr.). *Analysis.* Calc. for $C_7H_6N_2S$: S, 21.35. Found: S, 21.2.

Benzyl thiocyanate. From benzyl chloride and potassium thiocyanate in alcohol. The product melted at 41.0° (uncorr.). *Analysis.* Calc. for C_8H_7NS : S, 21.49. Found: S, 21.2.

β -thiocyanacetate of diethylene glycol monobutyl ether. Diethylene glycol monobutyl ether (Eastman, practical) was esterified with chloracetyl chloride in the presence of quinoline. The product was washed with dilute acid and alkali, and dried with anhydrous sodium sulphate. The solvent (benzene) was removed, and the product converted to the thiocyanogen compound by treatment with KSCN in alcohol. The product was not distilled for fear of decomposition, but the sulphur determination indicated a fairly pure substance. *Analysis.* Calc. for $C_{11}H_{13}NO_4S$: S, 12.27. Found: S, 12.43.

Phenacyl thiocyanate (thiocyanomethyl phenyl ketone). This compound was prepared from phenacyl bromide and KSCN. It melted at 73° (uncorr.). *Analysis.* Calc. for C_9H_7NOS : S, 18.10. Found: S, 17.8.

TOXICITY EXPERIMENTS

The compounds, the preparation of which is described above, were tested on *Aphis rumicis* using the method described in a previous publication (4). It was necessary to use a spreading agent with these compounds, and in the majority of the experiments Penetrol was used. The spray solutions were prepared by dissolving the compound to be tested in Penetrol, and diluting with water to make a spray containing 0.1 per cent of the compound, and 0.5 per cent of Penetrol. In a few cases a sulfonated fish oil (Tanoyl) was used as a spreader. Each experiment was run in duplicate, and a check experiment using the spreader alone was always performed. In these preliminary experiments the toxic agent was used at only one concentration because of the impracticability of running a large number of tests at the same time. In future work it is intended to test the more promising compounds at various concentrations.

The results of these tests are presented in Table I. Inspection of these figures shows that certain thiocyanogen compounds exhibit very high toxicity. It is also important to note that in the two cases where comparisons were made the toxicity of the thiocyanate greatly exceeded that of the corresponding halogen compound from which it was made. Evidently the introduction of the SCN group has conferred toxicity on an otherwise non-toxic molecule; but not all compounds containing this group are toxic. Methyl thiocyanacetate and *p*-thiocyanoaniline caused severe injury and β -thiocyanoethyl phenyl ether caused slight injury to nasturtium plants. Benzyl thiocyanate and thiocyanomethyl phenyl ketone formed poor emulsions. Considering both toxicity to *Aphis rumicis* and injury to foliage the most satisfactory compound was γ -thiocyanopropyl phenyl ether, which gave excellent control at this concentration (0.1 per cent) with no evidence of injury.

TABLE I

COMPARATIVE TOXICITY TO APHIS RUMICIS OF ORGANIC THIOCYANOGEN COMPOUNDS

Formula	Name	Per cent dead	
		Com- pound + spreader	Spreader alone
$ \begin{array}{ccccccc} & \text{H} & \text{H} & \text{H} & \text{H} & & \\ & & & & & & \\ \text{H} & - \text{C} & - \text{C} & - \text{C} & - \text{C} & - \text{SCN} \\ & & & & & & \\ & \text{H} & \text{H} & \text{H} & \text{H} & & \end{array} $	<i>N</i> -Butyl thiocyanate	33.3 31.0	Tanoyl 23.1 22.5
$ \begin{array}{c} \text{SCN} \\ \\ \text{C}_6\text{H}_{11} \end{array} $	Phenyl thiocyanate	24.5 23.4	16.3 20.8
$ \begin{array}{c} \text{H} \\ \\ \text{H} - \text{C} - \text{SCN} \\ \\ \text{H} \end{array} $	Methyl thiocyanate	64.3 43.2	23.5 18.0
$ \begin{array}{ccccccc} & \text{H} & & & \text{H} & & \\ & & & & & & \\ \text{H} & - \text{C} & - \text{O} & - \text{C} & - \text{C} & - \text{SCN} \\ & & & & & & \\ & \text{H} & & \text{O} & \text{H} & & \end{array} $	Methyl thiocyanacetate	66.0 54.7	28.5 35.2
$ \begin{array}{ccccccc} & \text{H} & & & \text{H} & & \\ & & & & & & \\ \text{H} & - \text{C} & - \text{O} & - \text{C} & - \text{C} & - \text{Cl} \\ & & & & & & \\ & \text{H} & & \text{O} & \text{H} & & \end{array} $	Methyl chloracetate	29.3 38.8	—
$ \begin{array}{ccccccc} & \text{H} & \text{H} & & & \text{H} & \text{H} \\ & & & & & & \\ \text{H} & - \text{C} & - \text{C} & - \text{O} & - \text{C} & - \text{C} & - \text{C} - \text{SCN} \\ & & & & & & \\ & \text{H} & \text{H} & & \text{O} & \text{H} & \text{H} \end{array} $	Ethyl β -thiocyanopropionate	50.3 60.8	20.6 32.1
$ \begin{array}{ccccccc} & \text{H} & \text{H} & & & \text{SCN} & \text{H} \\ & & & & & & \\ \text{H} & - \text{C} & - \text{C} & - \text{O} & - \text{C} & - \text{C} & - \text{C} - \text{H} \\ & & & & & & \\ & \text{H} & \text{H} & & \text{O} & \text{H} & \text{H} \end{array} $	Ethyl α -thiocyanopropionate	53.0 46.2	—
$ \begin{array}{ccccccc} \text{SCN} & \text{H} & \text{H} & & & & \\ & & & & & & \\ \text{H} & - \text{C} & - \text{C} & - \text{C} & - \text{O} & - \text{C}_6\text{H}_{11} \\ & & & & & & \\ \text{H} & \text{H} & \text{H} & & & & \end{array} $	γ -thiocyanopropyl phenyl ether	98.0 93.7	Penetrol 28.8 22.2
$ \begin{array}{ccccccc} \text{Br} & \text{H} & \text{H} & & & & \\ & & & & & & \\ \text{H} & - \text{C} & - \text{C} & - \text{C} & - \text{O} & - \text{C}_6\text{H}_{11} \\ & & & & & & \\ \text{H} & \text{H} & \text{H} & & & & \end{array} $	γ -bromopropyl phenyl ether	25.3 34.3	—

TABLE I (Continued)

Formula	Name	Per cent dead	
		Compound + spreader	Spreader alone
$ \begin{array}{ccccccc} & \text{H} & \text{SCN} & \text{H} & & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} \\ \text{H} & - \text{C} & - \text{C} & - \text{C} & - \text{C} & - \text{C} & - \text{C} & - \text{C} & - \text{H} \\ & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} \end{array} $	2-thiocyanooctane	37.3 52.8	25.3 13.9
$ \begin{array}{ccccccc} & \text{SCN} & \text{H} & & & \text{H} & \text{H} \\ \text{H} & - \text{C} & - \text{C} & - \text{O} & - \text{C} & - \text{C} & - \text{H} \\ & \text{H} & \text{H} & & & \text{H} & \text{H} \end{array} $	β -thiocyanoethyl ethyl ether	71.0 71.0	47.2 34.3
$ \begin{array}{ccccccc} & \text{SCN} & \text{H} & & & \text{H} \\ \text{H} & - \text{C} & - \text{C} & - \text{O} & - \text{C} & - \text{H} \\ & \text{H} & \text{H} & & & \text{H} \end{array} $	β -thiocyanoethyl methyl ether	86.7 86.5	16.2 21.3
$ \begin{array}{c} \text{NH}_2 \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{SCN} \end{array} $	<i>p</i> -thiocyanoaniline	99.2 99.1	24.1 28.6
$ \begin{array}{c} \text{CH}_2\text{SCN} \\ \\ \text{C}_6\text{H}_5 \end{array} $	Benzyl thiocyanate	91.7 81.6	20.0 26.9
$ \text{CH}_2\text{SCN} - \text{C}(=\text{O}) - \text{C}_6\text{H}_5 $	Thiocyanomethyl phenyl ketone	65.0 69.4	42.8 47.3
$ \begin{array}{c} \text{H} \qquad \qquad \text{H} \\ \qquad \qquad \\ \text{H} - \text{C} - \text{O} - \text{C} - \text{C} - \text{SCN} \\ \qquad \qquad \qquad \\ \text{H} \qquad \qquad \text{O} \qquad \text{H} \\ \diagup \qquad \diagdown \\ \text{H} \qquad \qquad \text{O} \\ \diagdown \qquad \diagup \\ \text{H} \qquad \qquad \text{C} \\ \qquad \qquad \\ \text{H} - \text{C} - \text{O} - \text{C} - \text{C} - \text{C} - \text{C} - \text{H} \\ \qquad \qquad \qquad \qquad \qquad \\ \text{H} \qquad \qquad \text{H} \qquad \text{H} \qquad \text{H} \qquad \text{H} \end{array} $	β -thiocyanacetate of diethylene glycol monobutyl ether	92.9 93.4	43.3 42.6
$ \begin{array}{ccccccc} & \text{SCN} & \text{H} & & & & \\ \text{H} & - \text{C} & - \text{C} & - \text{O} & - \text{C}_6\text{H}_5 \\ & \text{H} & \text{H} & & & & \end{array} $	β -thiocyanoethyl phenyl ether	93.6 94.5	43.8 45.8

ADDITIONAL EXPERIMENTS WITH γ -THIOCYANOPROPYL PHENYL ETHER

In order to test further the value of this compound experiments were performed on mealy bug (*Pseudococcus citri* Risso) and the red spider mite (*Tetranychus telarius* L.) using the material at a concentration of 0.1 per cent with Penetrol (0.5 per cent). Young coleus (*Coleus blumei* Benth.) plants badly infested with mealy bug were sprayed with this solution, and a kill of 98.0 per cent was obtained with 335 individuals counted without injury to the plants. Red spider mite on rose (*Rosa* sp.) with 250 individuals counted gave a kill of 100.0 per cent, without injury to the plants.

The following plants have been sprayed with the solution in order to obtain further information regarding tolerance: nasturtium (*Tropaeolum minus* L.), petunia (*Petunia axillaris* BSP.), English ivy (*Hedera helix* L.), eggplant (*Solanum melongena* L. var. *esculentum* L.), balsam (*Impatiens balsamina* L.), Jerusalem cherry (*Solanum pseudocapsicum* L.), cabbage (*Brassica oleracea* L. var. *capitata* L.), potato (*Solanum tuberosum* L.), peach seedlings (*Prunus persica* [L.] Stokes), shining club-moss (*Lycopodium lucidulum* Michx.), geranium (*Pelargonium* sp.), buckwheat (*Fagopyrum esculentum* Moench), gladiolus (*Gladiolus* sp.), African marigold (*Tagetes erecta* L.), cosmos (*Cosmos bipinnatus* Cav.), salvia (*Salvia splendens* Ker.), heliotrope (*Heliotropium peruvianum* L.), and cotton (*Gossypium hirsutum* L.). With the exception of buckwheat, no injury to the foliage was observed on any of the plants tested.

MODE OF ACTION ON INSECTS

Taubmann (15) has shown that the aliphatic thiocyanates are paralytic poisons affecting the cerebral axis of both warm-blooded and cold-blooded animals.

In addition to the toxicity experiments described above observations were made on the physiological response produced by external applications on the cockroach (*Periplaneta americana* L.), and a histological study of the ventral nerve cord of meal worm larvae (*Tenebrio molitor* L.), for the detection of nerve lesions.

Because of its large size the cockroach was selected for observations on the symptoms produced by γ -thiocyanopropyl phenyl ether. A drop of this compound was placed on the dorsum of a fifth instar nymph near the base of the abdomen. Fifteen minutes later it was noted that the individual carried the abdomen to one side and attempted to rub off the drop against the side of the cage. Longitudinal convulsive twitchings of the body and partial paralysis of the posterior pair of legs were the next symptoms noted. Within an hour after treatment paralysis of the legs was complete. Longitudinal twitchings of the body occurred at the rate of about one per second and the individual was unable to walk even when probed. From this time on there was a gradual decrease in convulsive

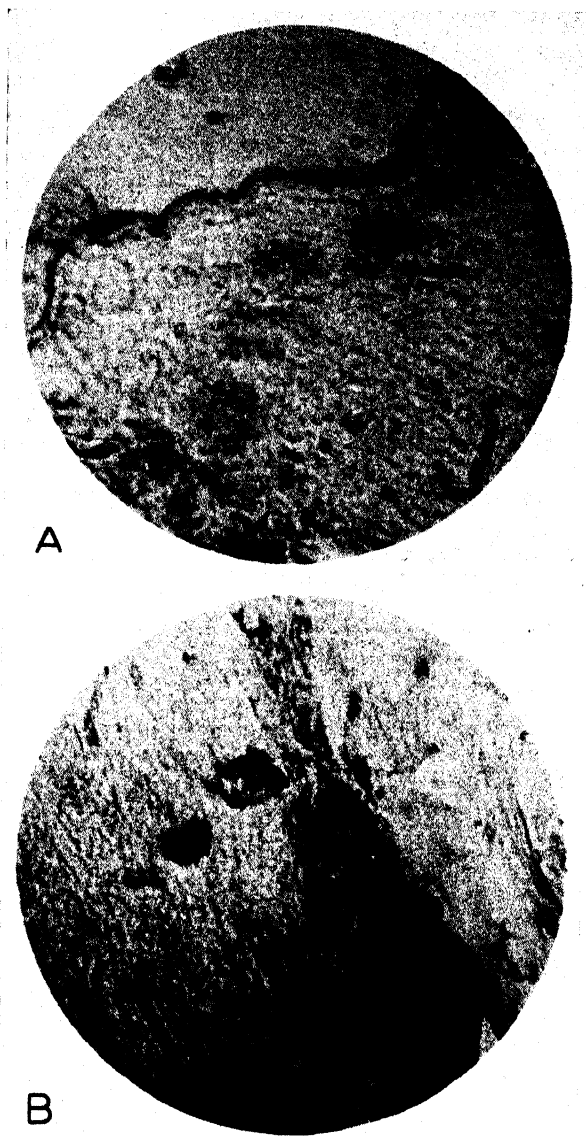


FIGURE 1. Effect of γ -thiocyanopropyl phenyl ether on meal worm larvae (*Tenebrio molitor*) ventral nerve ganglion. Cross section of ventral nerve ganglia stained with toluidine blue. $\times 1600$. (A) Larva killed by decapitation; (B) Larva killed by γ -thiocyanopropyl phenyl ether. Note the disintegration of the tissue; the violet-stained areas appear black in the photomicrograph.

movements and twitching of the legs. The individual was dead approximately three hours after treatment.

The symptoms described above, which are typical for a number of observations made in this study, suggested that the active agent is a nerve poison. In order to study the effect of this compound on the nervous system of insects applications were made on the dorsal surface of living meal worm larvae. In a number of cases the larvae appeared to be dead five minutes after treatment. All were subjected to treatment for 16 hours to assure death, after which they were carefully rinsed with acetone. The controls were killed by decapitation. The ventral nerve cord was dissected from each larva, fixed and stained with toluidine blue following a histological technique previously published (3) for the detection of paralysis. Cross sections of the abdominal ganglia of larvae killed by γ -thiocyanopropyl phenyl ether showed cellular degeneration which was indicated by violet-stained cell aggregates which appear black in the photomicrograph (Fig. 1, compare A and B). There was tigrolysis and vacuolization of the tissue but not in such a marked degree as with larvae killed by pyrethrum concentrates. Cross sections of the controls showed a homogeneous structure that stained uniformly blue throughout.

These histopathological studies are in accord with Taubmann's clinical observations on the central action of the aliphatic thiocyanates referred to above. Destruction of nerve tissue of the central nervous system appears to be a primary factor in bringing about paralysis and death in insects killed by γ -thiocyanopropyl phenyl ether.

SUMMARY

A preliminary study has been made of the toxicity of fifteen organic thiocyanogen compounds to *Aphis rumicis* on nasturtium, when used as spray materials. The compounds used included both aliphatic and aromatic compounds of various types. Several of the compounds exhibited marked toxicity to *Aphis rumicis* at the concentration used (0.1 per cent), much greater than that of the corresponding halogen compounds.

In several cases injury to nasturtium plants was noted. When injury and toxicity are considered, the most satisfactory compound was γ -thiocyanopropyl phenyl ether. This compound was further tested on mealy bug (*Pseudococcus citri*) and red spider mite (*Tetranychus telarius*), giving excellent control. Out of twenty species of plants sprayed with this compound at a concentration of 0.1 per cent, only buckwheat showed injury.

Observation of the symptoms produced on the cockroach (*Periplaneta americana*) by this compound indicated that it possessed the properties of a paralytic agent.

Nerve lesions were noted in meal worm larvae (*Tenebrio molitor*) that had been killed by γ -thiocyanopropyl phenyl ether applied externally

following a technique used in the detection of paralysis, involving the use of toluidine blue. Death is caused apparently by injury to the central nervous system accompanied by paralysis.

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THE EFFECT OF THE ALKYL HALIDES ON THE RESPIRATION OF POTATO TUBERS¹

LAWRENCE P. MILLER

The treatment of potato tubers (*Solanum tuberosum* L.) with chemicals markedly affects the dormancy and various metabolic processes of the tubers (6, 7, 8, 9, 10, 11). One of the most interesting of the effects obtained is the pronounced action of some of the chemicals in stimulating or retarding the respiratory activity of the tubers as measured by the CO₂ output (14). Thus when potato tubers are exposed to ethylene chlorhydrin vapor the CO₂ output is doubled within twelve hours after the start of the treatment (15) and reaches a value from three to eight times that of the control in about 50 hours, although treatment with the chemical is discontinued after 24 hours. Ethyl alcohol vapor on the other hand brings about a diminution of the CO₂ production within a few hours after the beginning of the treatment and a value as low as one-tenth that of the control may be reached a number of hours later. Increases of several hundred per cent have also been found to result from treatments with ethylene bromohydrin, ethyl mercaptan, acetaldehyde, and hydrocyanic acid, and decreases in the CO₂ output have been found to occur with treatments with methyl and *iso*-propyl alcohol (14).

It has been reported by McCallum (13) and Denny (6) that a number of the simpler hydrocarbon halides have some dormancy-breaking action when applied in very low concentrations. It was thought of interest to see whether these compounds affected the respiration and to learn whether, when a group of closely related compounds is considered, the effect on the respiration is correlated with the effect on dormancy. Previous work had indicated that one cannot predict the effect of a chemical in breaking the rest period from a knowledge of its influence on the respiratory activity of the tubers during the treatment period and for some time thereafter.

The results of the experiments discussed in the present paper have shown that the halogen substitution products of the hydrocarbons are very active in increasing the CO₂ output of potato tubers. Experiments with a considerable number of the ethyl, propyl, butyl, and amyl halides are reported. Interesting differences have been found between isomeric and otherwise closely related compounds. Determinations made over a range of concentrations for some of the compounds studied show that these substances differ not so much with regard to the increase in the CO₂ output which they can induce but rather with respect to the amount of the chemical necessary to bring about the increase.

¹ Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 76.

The normal alkyl halides have been found to be much more active than the corresponding secondary compounds, especially in the case of the bromides. The chlorides are much less active than the bromides, while the iodides are just as active or perhaps slightly more active than the bromides. The relative reactivity of some of the substances has been found to agree quite well qualitatively with the relative reactivity of these compounds as measured in certain chemical reactions.

A close correlation between the increase in respiratory activity induced by a treatment and its effect on the breaking of dormancy was not observed.

MATERIALS AND METHODS

The treatments were made by subjecting the tubers to the vapor from a definite amount of chemical applied on cotton placed in a closed container with the tubers for a period of 24 hours. At the end of 24 hours a stream of CO_2 -free air was drawn through the containers (three-liter desiccators were used for most of the work), and through Van-Slyke Cullen tubes containing standard $\text{Ba}(\text{OH})_2$ solution, in which the CO_2 from the respiring tubers was collected. Details of this method have been given previously (14). From the total amounts of CO_2 given off during various intervals the average rate per 100 grams per hour could be calculated. In most of the experiments here reported the measurements were confined to the first 48 hours since, as can be seen from the tables, the maximum effect of these chemicals is usually evident within 48 hours. The average rate of CO_2 production was calculated from the total amount of CO_2 obtained during two or three intervals within the 48 hours; the first period covered the time from the beginning of the treatment to 26 hours, comprising, therefore, the 24-hour treatment period and two hours thereafter, and the remaining period or periods covered the interval between 26 and 48 hours from the start of treatment. For purposes of comparison between various treatments the values obtained for the total amount of CO_2 given off by 100 grams tissue in 48 hours were used.

The determinations were made at 26°C ., the desiccators with the tubers being kept at this temperature by a thermostatically controlled water bath. In all the experiments samples of 750 grams each were used for the treatments. From experience in determining the respiratory activity of potato tubers it is known that a sample of this size furnishes a satisfactory sample of the tubers and that its CO_2 output can be determined quite accurately (14). A good estimate of the concordance of the results can be obtained from the tables. Many of the treatments were repeated a number of times and all the values obtained are shown. Variations are due not so much to errors in the methods of determination or to sampling errors, but to variation in the tubers occurring with age. Thus the CO_2 output of freshly-harvested tubers is much higher than after a short period of stor-

age. Also the tubers as they age become somewhat less responsive to the chemical treatments. Potatoes during the first few months after harvest are, however, quite satisfactory for these studies and do not change enough to make the interpretations of results difficult, since the differences between the activity of some of these alkyl halides are very large.

At the end of 48 hours when the respiration determinations were ended the tubers were cut up into pieces with one eye each and planted in flats in order to find out the effect of the treatment on dormancy and also to see whether the treatment had injured the tubers so as to bring about decay.

Several lots of tubers were used for these experiments. Potatoes of the Irish Cobbler variety grown in the Institute gardens, second crop Irish Cobbler potatoes obtained from New Jersey, and Bliss Triumph potatoes from Bermuda purchased in the Yonkers market were used. In general potatoes from these different lots gave similar results; differences were evident with regard to the amount of increase a given treatment produced, but relative increases from different chemicals were very much the same for different lots of tubers.

The chemicals used were obtained from the Eastman Kodak Company and the grade as listed in their catalog was that of the "highest purity."

RESULTS

With alkyl bromides. The results obtained in the determinations of the effect of the vapors of ethyl bromide, the two propyl bromides, the four butyl bromides, and *n*-amyl and *iso*-amyl bromides on the CO₂ output of Irish Cobbler potatoes from the Institute gardens are given in Tables I and II. It is seen from Table I that when potato tubers are exposed to the vapor from 0.05 cc. (per liter of air space in the container) of either ethyl bromide or *n*-propyl bromide the CO₂ output per 100 g. in 48 hours is increased to a value between three and four times that of the corresponding controls, the ethyl bromide and *n*-propyl bromide being quite similar in the magnitude of the effect produced. But secondary propyl bromide when applied in the same concentration only brings about an increase one-fifth as large as the isomeric normal or primary propyl bromide. By increasing the amount of the secondary bromide to 0.40 cc. per liter an increase in the CO₂ produced as large as with 0.05 cc. of the primary compound results. Thus the secondary compound is only about one-fifth to one-eighth as effective as the isomeric primary bromide. It will be noted that in the case of the primary bromide the increase resulting from 0.05 cc. per liter is near the maximum that can result from treatment with this chemical since higher concentrations up to 0.20 cc. per liter did not cause further increases.

The data in Table II show that treatments with 0.05 cc. per liter of

TABLE I

EFFECT OF ETHYL BROMIDE AND OF THE PROPYL BROMIDES ON THE RESPIRATION OF POTATO TUBERS (VARIETY IRISH COBBLER GROWN IN GARDENS OF BOYCE THOMPSON INSTITUTE)

Bromide used	Concn. cc. per l., 24 hrs.	Respiratory activity				
		Hours from beginning of treatment	Mg. CO ₂ per 100 g. per hr.		Mg. CO ₂ per 100 g. in 48 hrs.	
			Tr.*	Ck.*	Tr.	Ck.
Ethyl bromide	0.05	0-26.0	1.81	0.67	164.8	42.1
		26.0-43.5	5.35	1.18		
		43.5-48.0	5.34	0.92		
	0.05	0-26.0	1.52	0.48	145.8	30.8
		26.0-43.8	4.86	0.86		
		43.8-48.0	4.74	0.73		
Normal propyl bromide	0.05	0-26.0	1.76	0.51	142.3	32.6
		26.0-43.9	4.48	0.86		
		43.9-48.0	3.95	0.95		
	0.05	0-26.0	1.49	0.57	116.8	34.4
		26.0-43.3	3.66	0.90		
		43.3-48.0	3.16	0.87		
	0.10**	0-26.0	1.65	0.64	145.9	36.6
		26.0-47.8	4.66	0.91		
	0.20**	0-26.0	1.68	0.64	140.6	36.6
		26.0-48.0	4.41	0.91		
Secondary propyl bromide	0.05	0-26.0	0.69	0.51	53.7	32.6
		26.0-44.0	1.59	0.86		
		44.0-48.0	1.79	0.95		
	0.05	0-26.0	0.67	0.57	45.6	34.4
		26.0-43.3	1.19	0.90		
		43.3-48.0	1.62	0.87		
	0.10**	0-26.0	0.80	0.75	66.5	36.9
		26.0-48.0	2.08	0.79		
	0.20**	0-26.0	1.16	0.75	92.2	36.9
		26.0-48.0	2.82	0.79		
	0.40**	0-26.0	1.49	0.75	124.7	36.9
		26.0-48.0	3.90	0.79		
	0.80**	0-26.0	1.42	0.64	130.4	36.6
		26.0-48.0	4.25	0.91		

* Tr. = Tubers exposed to vapor of chemical; Ck. = Control tubers in air.

** These tests were conducted at room temperature.

normal butyl, and normal amyl and *iso*-amyl bromide increase the CO₂ output to about the same extent as ethyl and *n*-propyl bromide. Secondary butyl bromide proved to be much less effective than the normal bromide to a degree quite similar to the difference between *n* and secondary propyl

TABLE II

EFFECT OF THE BUTYL BROMIDES AND OF NORMAL AMYL AND ISO-AMYL BROMIDES ON THE RESPIRATION OF IRISH COBBLER POTATO TUBERS

Bromide used	Concn. cc. per l., 24 hrs.	Respiratory activity				
		Hours from beginning of treatment	Mg. CO ₂ per 100 g. per hr.		Mg. CO ₂ per 100 g. in 48 hrs.	
			Tr.	Ck.	Tr.	Ck.
Normal butyl bromide	0.05	0-26.0	1.96	0.82	123.4	45.2
		26.0-43.9	3.36	1.09		
		43.9-48.0	2.95	1.09		
	0.05	0-26.0	1.67	0.65	101.4	38.5
		26.0-43.7	2.74	1.02		
		43.7-48.0	2.19	0.84		
	0.05	0-26.0	1.80	0.63	124.9	38.8
		26.0-43.8	3.38	0.98		
		43.8-48.0	4.26	1.19		
	0.10	0-26.0	1.64	0.63	137.8	38.8
		26.0-43.8	4.26	0.98		
		43.8-48.0	4.57	1.19		
Iso-butyl bromide	0.05	0-26.0	1.72	0.65	138.4	37.3
		26.0-48.0	4.26	1.02		
	0.05	0-26.0	1.27	0.77	86.9	43.9
		26.0-43.6	2.54	1.10		
		43.6-48.0	2.08	1.04		
	0.05	0-26.0	0.63	0.60	50.4	41.4
		26.0-43.0	1.17	1.07		
		43.0-48.0	2.85	1.07		
	0.05	0-26.0	0.84	0.65	56.3	38.5
		26.0-43.7	1.59	1.02		
		43.7-48.0	1.51	0.84		
Secondary butyl bromide	0.20	0-26.0	1.34	0.65	99.2	37.3
		26.0-48.0	2.92	1.02		
	0.05	0-26.0	1.06	0.48	67.6	30.8
		26.0-43.8	1.88	0.86		
		43.8-48.0	1.54	0.73		
	0.05	0-26.0	1.44	0.57	84.8	34.0
		26.0-43.7	2.10	0.92		
		43.7-48.0	2.40	1.00		
	0.05	0-26.0	1.62	0.60	100.7	32.4
		26.0-42.8	2.93	0.77		
		42.8-48.0	2.30	1.24		
Normal amyl bromide	0.10	0-26.0	1.54	0.60	102.6	33.4
		26.0-42.8	2.76	0.77		
		42.8-48.0	4.14	1.24		
	0.05	0-26.0	1.71	0.57	103.7	34.0
		26.0-43.7	2.78	0.92		
		43.7-48.0	2.36	1.00		
	0.10	0-26.0	1.79	0.60	115.3	33.4
		26.0-42.8	2.90	0.77		
		42.8-48.0	5.44	1.24		
	0.05	0-26.0				
		26.0-43.7				
		43.7-48.0				
Iso-amyl bromide	0.05	0-26.0				
		26.0-43.7				
		43.7-48.0				
	0.10	0-26.0				
		26.0-42.8				
		42.8-48.0				
	0.05	0-26.0				
		26.0-43.7				
		43.7-48.0				
	0.10	0-26.0				
		26.0-42.8				
		42.8-48.0				

bromides; 0.05 cc. per liter of the secondary compound resulted in an increase in CO_2 output of only one-fifth of that resulting from 0.05 cc. per liter of the primary bromide. The increase in CO_2 output resulting from *iso*-butyl bromide showed this compound to be more similar to the normal compound than to the secondary halide, the *iso* compound causing an increase one-half of that produced by the normal. In the case of *iso*-amyl bromide, in which the branched chain is farther from the halogen end of the molecule, the effect on the respiration was still more like that of the normal compound so that at the concentrations studied the *iso* and normal amyl bromides seem to be equally efficacious. The tertiary butyl bromide was found to be intermediate in its effectiveness between the *iso* and secondary compound.

Those results showing the importance of isomerism in the response of potato tubers to the alkyl bromides were further checked by repeating the treatments, using 0.05 cc. per liter in each case, with another lot of tubers. These tubers gave larger increases as a result of the treatments than the tubers used in the experiments reported in Tables I and II. The data obtained (Table III) show qualitative agreement with the experiments with the other lot of tubers with the exception of the treatments with ethyl bromide which were somewhat less effective in these tests than in the previous experiments. The effects were, however, of the same order of magnitude as those of the other normal bromides. Due presumably to the greater stimulation that could be brought about in this lot of tubers, the differences between the effects of the normal and secondary propyl and butyl bromides were even greater than in the case of the previous treatments. The relative activity of the bromides at this concentration will be further discussed in a later section of the paper (Table VI).

With alkyl chlorides and iodides. The results of experiments on the effect on respiration of some of the alkyl chlorides and iodides as compared with the bromides are tabulated in Table IV. Horizontal lines separate different experiments, consisting of a number of treatments and a control, from each other, and the tests run on the same lot of tubers have been placed adjacent to each other. Many of the chemicals were applied in a series of concentrations, thus furnishing a better basis for comparisons between chemicals than if the effect of only one concentration in each case is known.

The small quantities which it was necessary to measure out in some of the cases (three-liter containers were used) were obtained by the use of a 0.2 cc. micro-pipette calibrated in thousandths of a cc. In the various tests the amounts of chemical added were regulated in terms of cc. of liquid rather than in molar quantities. In the case of the substances studied, the normal propyl and butyl chlorides, bromides, and iodides, the differences in specific gravity compensate to a large degree for the difference in molecular weight so that a comparison between the effect

TABLE III

EFFECT OF ALKYL BROMIDES ON THE RESPIRATION OF POTATO TUBERS (VARIETY IRISH COBBLER GROWN IN SOUTHERN NEW JERSEY)

Alkyl radical	Respiratory activity					Average increase in CO ₂ output in 48 hrs.
	Hours from beginning of treatment	Mg. CO ₂ per 100 g. per hr.		Mg. CO ₂ per 100 g. in 48 hrs.		
		Tr.	Ck.	Tr.	Ck.	
Ethyl	0-26	2.10	0.66	183.7	39.5	138.2
	26-48	5.88	1.02			
	0-26	2.50	0.87	185.7	52.2	
	26-48	5.48	1.35			
	0-26	2.29	0.87	189.0	52.2	
	26-48	5.89	1.35			
Normal propyl	0-26	2.59	0.66	215.4	39.5	189.1
	26-48	6.73	1.02			
	0-26	3.30	0.89	259.1	56.9	
	26-48	7.87	1.53			
Secondary propyl	0-26	0.72	0.66	46.0	39.5	6.5
	26-48	1.24	1.02			
Normal butyl	0-26	3.10	0.92	206.5	60.1	130.1
	26-48	6.10	1.64			
	0-26	2.96	0.66	192.7	39.5	
	26-48	5.26	1.02			
	0-26	2.24	0.66	131.4*	39.5	
	26-48	3.32	1.02			
	0-26	2.88	0.87	181.1	52.2	
	26-48	4.84	1.35			
Iso-butyl	0-26	2.44	0.92	138.9	60.1	84.3
	26-48	3.43	1.64			
	0-26**	2.45	0.87	142.0	52.2	
	26-48	3.56	1.35			
Secondary butyl	0-26	1.16	0.92	74.7	60.1	14.6
	26-48	1.99	1.64			
Tertiary butyl	0-26	1.76	0.92	95.2	60.1	35.1
	26-48	2.26	1.64			
	0-26**	1.51	0.87	87.2	52.2	
	26-48	2.18	1.35			
Normal amyl	0-26	2.49	0.66	146.8	39.5	107.3
	26-48	3.72	1.02			
Iso-amyl	0-26	2.48	0.66	150.5	39.5	111.5
	26-48	3.90	1.02			

* This figure is obviously too low. The reason for the abnormal value obtained in this case is not known.

** These treatments were made with redistilled material. The *iso*-butyl bromide boiled between 91.6° and 92.0°C., the tertiary butyl bromide between 73° and 73.5°C.

TABLE IV
EFFECT OF ALKYL HALIDES ON THE RESPIRATION OF POTATO TUBERS

Alkyl halide	Concn. cc. per l., 24 hrs.	Mg. CO ₂ per 100 g. per hour during various periods after start of treatment		Mg. CO ₂ per 100 g. in. 48 hrs.	Increase over control
		0-26 hrs.	26-48 hrs.		
<i>n</i> -Butyl chloride	0.05	0.59	1.08	39.1	0.3
<i>n</i> -Butyl iodide	0.05	1.66	3.08	111.0	72.2
Control		0.63	1.02	38.8	Control
<i>n</i> -Amyl chloride	0.05	0.64	1.35	44.5	11.1
<i>n</i> -Amyl iodide	0.05	1.40	2.44	87.6	54.2
Control		0.60	0.88	33.4	Control
<i>n</i> -Butyl chloride	0.10	0.59	1.08	38.7	4.3
<i>n</i> -Amyl chloride	0.10	0.65	2.16	64.6	30.2
Control		0.57	0.89	34.4	Control
Normal propyl iodide	0.20†	2.06	4.70	156.8	107.8
	0.10	1.87	4.42	145.8	96.8
	0.05	1.72	3.53	122.3	73.3
Secondary propyl iodide	0.20	1.66	4.04	132.0	83.0
	0.10	1.67	3.98	131.0	82.0
	0.05	1.31	2.64	92.1	43.1
Control		0.85	1.22	49.0	Control
Normal propyl chloride	1.60†	2.46	6.96	217.2	169.2
	0.80	1.98	5.96	186.9	138.9
	0.20	0.87	1.61	57.1	9.1
	0.05	0.81	1.12	45.5	-2.5
Secondary propyl chloride	1.60†	1.91	5.14	162.8	114.8
	0.80	1.50	3.64	119.0	71.0
	0.20	0.73	1.21	45.7	-2.3
	0.05	0.73	1.26	46.8	-1.2
Control		0.79	1.24	48.0	Control
Normal propyl bromide	0.05	3.83	4.39	196.1	107.2
	0.025	3.12	—	—	—
	0.013	2.47	3.16	133.7	44.8
	0.0063	1.88	2.82	110.8	21.9
Normal propyl iodide	0.05	3.86	6.04	233.5	144.6
	0.025	3.39	3.79	171.6	82.7
	0.013	2.66	2.95	134.2	45.3
	0.0063	2.12	2.36	107.0	18.1
Control		1.52	2.24	88.9	Control
<i>n</i> -Butyl chloride	0.60†	2.84	6.25	211.3	149.6
	0.20	2.12	4.01	143.5	81.8
Normal butyl bromide	0.025	2.40	3.25	134.0	72.3
	0.013	1.73	2.56	101.0	39.3
	0.0063	1.29	1.91	75.5	14.0
Normal butyl iodide	0.025	2.50	3.28	137.0	75.3
	0.013	2.00	2.62	109.6	47.9
	0.0063	1.90	2.54	105.2	43.5
Control		0.97	1.66	61.7	Control

* Institute Irish Cobbler potatoes were used for these experiments; ** New Jersey Irish Cobbler potatoes were used in this experiment; *** Bermuda Bliss Triumph potatoes were used in these experiments.

† Some injury to the tubers resulted from this treatment.

of, for example, 0.05 cc. per liter of propyl chloride with 0.05 cc. per liter of the iodide is in fact a comparison between molar concentrations which are quite similar. The differences observed are so much larger than could be accounted for by the small differences in the molar concentration that the latter can be ignored in these measurements.

From the data of Table IV it is seen that the primary chlorides and iodides are also more active than the secondary compounds as was the case with the bromides previously considered. From the data available, which cover only the propyl compounds, it seems that these differences are not as large, especially in the case of the iodides, as with the bromides. The results of the first three experiments listed in the table show the effect on the respiration of 0.05 cc. and 0.10 cc. per liter of butyl and amyl chloride compared with 0.05 cc. per liter of the corresponding iodides. These experiments were conducted with tubers from the same lot as was used for the experiments shown in Table II. It is seen that the increases resulting from treatments with the iodides are about the same as those with the bromides, but the chlorides are much less effective. This difference between the efficacy of the bromides and iodides as compared with the chlorides is further brought out in the last experiment listed in Table IV, in which the *n*-butyl compounds were used. When the amounts are sufficiently increased much stimulation in respiration also results from treatments with the chlorides (see also the propyl chlorides in Table IV).

An attempt to determine more accurately any difference between the relative efficacy of the bromides and the iodides was made in the experiments the data of which are given in the last two sections of Table IV. It seems that normal propyl iodide produces somewhat greater stimulation than the same concentration of the bromide, and this is probably true of the butyl compounds also. It is of interest that in the case of normal propyl and butyl bromides and iodides definite stimulation of respiration occurs with concentrations as low as 0.0063 cc. per liter, or from 24 to 33 mg. of chemical per 750 grams of potato tubers.

The results obtained with the alkyl halides may be recapitulated as follows. When potato tubers are exposed for 24 hours to the vapor from 0.05 cc. per liter of ethyl, *n*-propyl, *n*-butyl, *n*-amyl, or *iso*-amyl bromides the CO₂ output is increased several hundred per cent. Secondary propyl and secondary butyl bromides when applied in the same concentration cause increases only about one-fifth of those resulting from the normal compounds. The effect of *iso*-butyl bromide was considerably greater than that of the secondary but less than that of the normal; tertiary butyl bromide brought about an increase intermediate between the secondary and *iso* compounds. The increases in CO₂ output resulting from treatment with the secondary compounds were greater when higher concentrations were used. In the case of the representatives tested (the propyl compounds)

the normal chlorides and iodides are also more active than the corresponding secondary compounds. Considerably higher concentrations of the chlorides (about five or more times higher) as compared with the bromides are necessary to cause large increases in the CO_2 output, while the iodides are as effective or perhaps slightly more effective than the bromides. Definite increases in the respiratory activity occurred with concentrations of bromide and iodide as low as 0.0063 cc. per liter of air space in the containers.

Effect of some substances that might be derived from the alkyl bromides on the respiration. Since such large differences have been found between the effects of some of the alkyl halides on respiration, attention is naturally directed toward a possible reason for these differences. With a view to a further understanding of the action of the alkyl bromides, with special reference to normal and secondary propyl bromides, the effect of a number of products that might be derived from the bromides on the CO_2 output was studied. The active agent in affecting the respiration may be the unaltered bromide or some substance derived from the bromide. Thus the bromides may undergo hydrolysis resulting in the formation of hydrogen bromide and the corresponding alcohol. Or bromine might be split off from the halide leaving unsaturated hydrocarbons. (Judging from the reagents necessary to bring about this reaction in the laboratory splitting off of bromine is much less likely to occur in the tubers than the simple hydrolysis mentioned above.) Results of some tests on the effect of hydrobromic acid, bromine, propylene, normal and *iso*-propyl alcohol, and the four butyl alcohols on respiration are given in Table V. Some of these chemicals were tested at concentrations near that of the unaltered bromide necessary to cause large increases, while others were tried at even much higher concentrations. Under the conditions of these experiments none of these chemicals produced increases as large as normal propyl bromide. It must be kept in mind, of course, that the fact that HBr , for example, does not cause an increase in respiration when put in the same container with the tubers does not prove that it may not act effectively when it is formed within the tuber as a result of the hydrolysis of some absorbed propyl bromide.

However, it is quite unlikely that differences in the activity of the bromides are due to the products of hydrolysis since the secondary and tertiary halides are much more easily hydrolyzed than the primary halides (16) and should, therefore, be much more effective unless the primary alcohols formed are more active than the secondary and tertiary alcohols resulting from the corresponding bromides. There is no evidence at present that there is any great difference between the efficacy of these various alcohols. In fact any stimulative action observed in the experiments with the alcohols has been slight.

TABLE V

EFFECT OF SOME SUBSTANCES THAT MIGHT BE OBTAINED FROM ALKYL BROMIDES
ON THE RESPIRATION OF POTATO TUBERS

Chemical	Concn. cc. per l., 24 hrs.	Mg. CO ₂ per 100 g. per hr. at various intervals after start of treatment		Mg. CO ₂ per 100 g. in. 48 hrs.
		0-26 hrs.	26-48 hrs.	
<i>n</i> -Propyl bromide	0.05	3.30	7.87	259.1
Propylene	14.0	1.58	2.18	89.0
Bromine	0.03	0.97	1.49	57.9
Control	—	0.89	1.53	56.9
<i>n</i> -Propyl bromide	0.05	2.36	5.58	184.6
Bromine	0.06	0.80	1.09	44.8
HBr	0.045*	1.01	1.54	58.8
HBr	0.091*	0.86	1.36	52.3
Control	—	0.72	1.21	45.6
<i>n</i> -Propyl alcohol	3.00**	1.49	2.16	86.5
	1.50	2.70	4.68	173.7
	0.75	2.52	5.06	176.7
<i>Iso</i> -propyl alcohol	3.00	0.81	1.72	58.8
	0.75	2.09	3.18	124.5
Control	—	1.71	2.86	107.4
<i>Iso</i> -propyl alcohol	3.00	0.36	0.42	18.6
	1.5	0.45	0.67	26.5
	0.75	0.65	1.13	41.8
	0.37	0.59	1.03	38.2
	0.18	0.61	0.99	37.7
Control	—	0.61	0.97	37.1
<i>n</i> -Butyl alcohol	2.0	0.63	—	—
<i>Iso</i> -butyl alcohol	2.0	0.67	1.18	43.3
<i>Sec.</i> -butyl alcohol	2.0	0.77	1.49	52.8
<i>Tert.</i> -butyl alcohol	2.0	0.49	0.94	33.4
Control	—	0.64	1.01	38.8
<i>n</i> -Butyl alcohol	1.0	0.71	1.39	49.2
<i>Iso</i> -butyl alcohol	1.0	0.81	1.39	41.6
<i>Sec.</i> -butyl alcohol	1.0	0.64	1.09	40.5
<i>Tert.</i> -butyl alcohol	1.0	0.71	1.42	51.0
Control	—	0.57	0.90	34.6
<i>n</i> -Butyl alcohol	0.5	0.76	1.72	57.6
<i>Iso</i> -butyl alcohol	0.5	0.57	1.08	38.6
<i>Sec.</i> -butyl alcohol	0.5	0.54	1.06	37.4
<i>Tert.</i> -butyl alcohol	0.5	0.55	1.14	39.4
Control	—	0.59	0.95	36.3

* Grams of HBr in 40 per cent solution per liter.

** This treatment produced considerable injury.

Relative reactivity of the alkyl halides. It is well known that in various chemical reactions the chlorides are much less active than the bromides and the bromides less active than the iodides. Thus the chlorides will not react with AgNO_3 to form a precipitate of silver halide, but the bromides react at the boiling temperature and the iodides in the cold (3, p. 58). The results of the studies on the effect on respiration agree well with this order of reactivity except that the differences noted in the efficacy between the iodides and bromides are smaller than one might expect from the difference observed in various chemical reactions. Now as to the differences among the isomers and homologs within the chlorides, bromides, or iodides, much work has been done in determining the relative reactivity in various chem-

TABLE VI

RELATIVE EFFECTIVENESS OF THE ALKYL BROMIDES IN INCREASING THE CO_2 OUTPUT COMPARED WITH THE RELATIVE REACTIVITY OF THE ALKYL HALIDES IN SOME CHEMICAL REACTIONS

Alkyl radical	Institute Irish Cobblers		New Jersey Irish Cobblers		Relative rate of formation of alkyl pyridinium bromide**	Relative reactivity of the chlorides†
	Increase in CO_2 output	Relative efficacy*	Increase in CO_2 output	Relative efficacy*		
Ethyl	113.9	1.50	138.2	1.06	2.10	1.9-2.5
<i>n</i> -Propyl	96.1	1.27	189.1	1.45	1.40	1.0-1.1
<i>Sec.</i> -propyl	16.6	0.22	6.5	0.05	0.14	0.015
<i>n</i> -Butyl	75.6	1.00	130.1	1.00	1.00	1.00
<i>Iso</i> -butyl	43.0	0.57	84.3	0.65	0.07	—
<i>Sec.</i> -butyl	13.4	0.18	14.6	0.11	0.09	0.022
<i>Tert.</i> -butyl	36.8	0.49	35.1	0.27	—	0.018
<i>n</i> -Amyl	59.5	0.79	107.3	0.83	—	1.31
<i>Iso</i> -amyl	69.7	0.92	111.5	0.86	—	0.65

* Relative efficacy of 0.05 cc. per l. which represents the following relative molar quantities: ethyl 1.43, *n*-propyl 1.13, *sec.*-propyl 1.08, *n*-butyl 1.00, *iso*-butyl 0.99, *sec.*-butyl 1.00, *tert.*-butyl 1.00, *n*-amyl 0.87, *iso*-amyl 0.87.

** Data of Noller and Dinsmore (17).

† Relative reactivity in reaction $\text{RCl} + \text{KI} \rightarrow \text{RI} + \text{KCl}$. Data of Conant and Hussey (4).

ical reactions. As already indicated, in some reactions, as for example, hydrolysis to hydrobromic acid and alcohol, the secondary and tertiary compounds are much more reactive than the primary. [See also Noller and Dinsmore (17, p. 1031).] There are, however, other reactions in which the order of reactivity is reversed, the normal being more reactive than the secondary. No attempt will be made here to review all of this literature but a few results from recent papers will be given. The relative rate of the formation of alkyl pyridinium bromide when the alkyl bromides react with pyridine as determined by Noller and Dinsmore (17) and the relative reactivity of the alkyl chlorides in the reaction $\text{RCl} + \text{KI} \rightarrow \text{RI} + \text{KCl}$ from the data of Conant and Hussey (4) are shown in columns 6 and 7 of Table VI. For comparison the relative increases in the CO_2 pro-

duced in 48 hours from treatments of potato tubers with 0.05 cc. per liter are shown for two lots of potatoes in columns 3 and 5. One is not justified in comparing too seriously these biological measurements with physico-chemical determinations in which many more factors are under control, but it is of interest to note the similarity between the results obtained with the potato tubers and in the chemical reactions. The qualitative agreement between the relative efficacy in increasing the respiration and in the chemical reactivity in the reactions considered is fair. In the potato experiments ethyl bromide was no more efficacious than propyl bromide at the concentration studied. A difference is also evident between the activity of *iso*-butyl and tertiary butyl bromides as compared with *n*-butyl bromide. As can be seen from Table III this difference cannot be ascribed to the presence of impurities since the same results were obtained after these preparations had been redistilled.

These comparisons between the effectiveness of the alkyl halides in increasing the respiratory activity of potato tubers and their relative reactivity in certain chemical reactions, of course, prove nothing with respect to the possible mechanism of their action. The results do suggest, however, that the stimulative action of these compounds is in some way closely related to their chemical reactivity.

RELATION BETWEEN EFFECT ON CO₂ OUTPUT AND EFFICACY IN BREAKING DORMANCY

A study of the relation between the effect of a treatment on the CO₂ output and its effect on dormancy is of interest since several workers consider that increases in respiration are important factors in connection with dormancy-breaking agents (1, 18). Results obtained in experiments on the effect of ethylene chlorhydrin, ethyl mercaptan, and ethyl alcohol, and of cutting of the tubers on the CO₂ output and on dormancy failed to show any close correlation between these two processes (14). Data obtained in connection with the present studies of the effect of alkyl halides on the CO₂ output confirm these conclusions.

In Table VII are shown the results of all the experiments on the Institute Irish Cobbler potatoes with the alkyl halides (some alkylene halides are also included) which produced increases of over 100 per cent in the CO₂ output and which were conducted while the tubers were still dormant enough so that it took 40 or more days for 50 per cent of the untreated controls to show sprouts above ground. The same tubers were used to obtain the germination data as had been used for the measurement of CO₂ production. The treatments are listed in decreasing order of their effectiveness in causing an increase in respiratory activity. Increases from 110 to 373 per cent were obtained. The figures for the number of days for 50 per cent of the planted pieces to show sprouts above ground are in every case

TABLE VII

RELATION BETWEEN INCREASE IN CO₂ OUTPUT AND EFFECT ON DORMANCY RESULTING FROM TREATMENTS OF POTATO TUBERS WITH CHEMICALS

Chemical	Concn., cc. per l., 24 hrs.	Mg. CO ₂ per 100 g. in 48 hrs.	No. of days for 50% to appear above ground	
		Ratio Tr./Ck.	Tr.	Ck.
Ethyl bromide	0.05	4.73	49	51
<i>n</i> -Propyl bromide	0.05	4.40	42	51
Propylene bromide	0.05	4.27	49	73
Ethyl bromide	0.05	3.90	68	69
Trimethylene bromide	0.05	3.57	65	69
Ethylene bromide	0.05	3.56	*	64
<i>n</i> -Butyl bromide	0.10	3.55	34	44
<i>Iso</i> -amyl bromide	0.10	3.48	33	40
<i>n</i> -Butyl bromide	0.05	3.22	33	44
<i>n</i> -Amyl bromide	0.05	3.08	38	40
<i>Iso</i> -amyl bromide	0.05	3.05	48	64
Methylene bromide	0.05	3.04	69	68
<i>n</i> -Amyl bromide	0.05	3.01	42	40
<i>n</i> -Butyl iodide	0.05	2.86	41	44
<i>n</i> -Butyl bromide	0.05	2.74	36	65
<i>n</i> -Butyl bromide	0.05	2.64	59	60
<i>n</i> -Amyl iodide	0.05	2.62	42	40
Ethylene chloride	0.05	2.60	36	44
<i>n</i> -Amyl bromide	0.05	2.50	60	64
β -Butylene bromide	0.05	2.32	45	64
<i>Tert.</i> -butyl bromide	0.05	2.20	52	51
Ethylene chlorhydrin**	1.00	1.21	30	79
Ethylene chlorhydrin†	1.00	3.22	30	79
Ethylene chlorhydrin**	1.00	2.20	27	60

* The tubers in this treatment rotted.

** These treatments were made with 1.0 cc. per l. of a solution made by adding 10 cc. of chlorhydrin to 90 cc. of ethyl alcohol.

† Ten per cent aqueous solution was used for this treatment.

the average of two lots of 20 pieces. The probable error of these values is ± 1.98 days and the probable error of the difference between the number of days for 50 per cent of the treated and check pieces to appear above ground is ± 2.79 days. A difference of nine days or more can therefore be considered significant. An examination of the data shows that although over half of the treatments caused increases of two hundred per cent and more in the CO₂ output only a few had any effect on the dormancy. Those treatments which brought about germination nine or more days earlier than in the control are not confined to the upper half of the table and are, therefore, not correlated with increase in CO₂ production.

For purposes of comparison three ethylene chlorhydrin treatments are listed at the bottom of the table. These were made on tubers from the same lot and during the same period as the experiments with the alkyl and alkylene halides. These treatments were made with 10 per cent solutions of the chlorhydrin in water or in alcohol. This concentration of the chlor-

hydrin is less than that usually used (40 per cent solution) and its effect on respiration was, therefore, somewhat less than that usually obtained, and in the case of the treatments in alcoholic solution the tendency of the chlorhydrin to increase the respiration was further retarded by the presence of the alcohol (15). The first two chlorhydrin treatments shown were made on the same day and are directly comparable. It is seen that the presence of the alcohol greatly lessened the effect of the chlorhydrin on the CO_2 production but did not interfere with its dormancy-breaking action. Thus, while ethylene chlorhydrin and alcohol are antagonistic with regard to their effect on the respiration (15) of potato tubers they are not antagonistic in their effect on dormancy. The three chlorhydrin treatments shown in the table were less effective on the CO_2 output than most of the treatments with the alkyl halides, yet they broke dormancy satisfactorily.

These results show that the effect of a chemical treatment on the CO_2 output of potato tubers during the period of treatment and for some time thereafter cannot give any indication of its effect on dormancy. If the effect of the chemical on respiration plays the determining part in connection with the breaking of dormancy it is not shown by the effects on the intact tubers during the treatment and for some time thereafter but must make itself manifest after the tubers are cut into pieces and planted.

DISCUSSION

It might be suggested that the increase in CO_2 output which results from treatments with chemicals is merely a response of the potato tubers to injury and that the chemicals which are effective in the lowest concentrations and which bring about the largest increases are those which are the most toxic. However, the effect of the chemicals cannot be explained on this basis. When potato tubers are exposed to sufficient quantities of the vapor of various chemicals to produce injury (injury being here understood to mean that the tubers rot as a result of the treatment) the respiratory response is not the same, either quantitatively or qualitatively, for all chemicals. The alkyl halides when applied in concentrations which had any effect at all always produced increases in the CO_2 output and in no cases were decreases observed even when lethal quantities were used. On the other hand methyl, ethyl, and the propyl alcohols, acetone, and the propyl ethers (the data obtained with acetone and the propyl ethers are unpublished) decrease the CO_2 output when high concentrations are used. Thus when potato tubers are killed by exposure to the vapor of various chemicals the CO_2 output may be greatly increased or greatly decreased depending upon the chemical used. When proper concentrations are employed large increases or large decreases may occur without any permanent injury to the tubers (i.e., the tubers remain perfectly sound when subsequently cut into pieces and planted in soil and on germination normal growth takes place).

Considerable work has been done on the effect of anaesthetics and other chemicals on plant respiration. No attempt will be made here to review this literature since the experiments were largely conducted on green leaves and other plant material quite different in character from potato tubers. Brief discussion of this work is given in a number of readily available reference works and in most texts on plant physiology (see e.g. 2; 5, p. 158; 12). It may be important to point out a few differences in the effects obtained with some of the chemicals used in our studies and those previously reported. In general the increases obtained with ethylene chlorhydrin and the alkyl halides are very large compared with those obtained with many of the chemicals previously studied. Also in a large number of the previous experiments it is reported that higher concentrations cause a fall in respiration below normal. In our experiments such a fall below normal was obtained only with the alcohols, acetone, and the propyl ethers while the alkyl halides, ethylene chlorhydrin, and a number of other chemicals have always produced increases even when lethal concentrations were used and the CO₂ measurements were continued until rotting was evident in the treated tubers.

The results in this paper together with some data previously published (14) yield information on the effect of methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *iso*-butyl, *tert.*-butyl, and *sec.*-butyl alcohols on the respiratory activity. Methyl, ethyl, and the propyl alcohols depress the respiration rather sharply when high concentrations are used. The butyl alcohols seem to be without any great effect even when concentrations of 4 cc. per liter are used (the results with 4 cc. per l. are omitted from Table V since this concentration did not cause any different effect from the lower concentrations). This is rather surprising in view of the great increase in the toxicity of the alcohols from methyl to butyl in animals (19, p. 715) and on potato tuber discs as determined by Stiles and Stirk (20), who used the effect on exosmosis as a measure of toxicity. It is quite probable that the lesser activity of the butyl alcohols in our experiments was due to the fact that they were not absorbed by the intact tubers as readily as the lower members of the series.

SUMMARY

Experiments were conducted in which potato tubers were exposed in closed containers to the vapor from various alkyl halides for 24 hours and the CO₂ given off during the 24-hour treatment period and for 24 hours thereafter determined. These compounds were found to be very active in increasing the CO₂ output and marked differences were observed in the effect of some isomeric and otherwise closely related compounds. The differences were evident not so much with regard to the amount of increase the various chemicals would induce but rather with respect to the concentration of chemical necessary to bring about the increase.

Treatments with 0.05 cc. per liter of ethyl, *n*-propyl, *n*-butyl, *n*-amyl, and *iso*-amyl bromides were about equally efficacious and resulted in increases of several hundred per cent in the CO₂ output. But secondary propyl bromide and secondary butyl bromide caused increases only about one-fifth as large as the isomeric normal bromides. When higher concentrations of these secondary compounds were used much stimulation in the CO₂ production also resulted. *Iso*-butyl bromide was about one-half as effective as the normal compound and the efficacy of tertiary butyl bromide was intermediate between that of the secondary and *iso* compounds.

When concentrations of 0.05 cc. per liter were used, the iodides were about as effective as the bromides, while the chlorides were much less effective. When the concentration of the chlorides was increased several times, large increases in CO₂ output resulted. A study of some members of the group in a range of concentrations showed that the secondary chlorides and iodides are also less effective than the corresponding normal compounds. Results with concentrations below 0.05 cc. indicate that the iodides may be somewhat more effective than the bromides. Definite increases were observed with some of the bromides and iodides with concentrations as low as 0.0063 cc. per liter.

The relative efficacy of the alkyl halides in increasing respiration is related to their relative reactivity in certain chemical reactions.

Tests with hydrobromic acid, bromine, propylene, *n*- and *iso*-propyl alcohol, and the four butyl alcohols, substances which might possibly be formed within the tubers from absorbed halides, have failed to show increases as large as those produced by the unaltered halides.

The effect of a particular chemical treatment on the respiratory activity is not correlated with its effect on dormancy.

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REVERSAL OF DIRECTION OF TRANSLOCATION OF SOLUTES IN STEMS

P. W. ZIMMERMAN AND MARY H. CONNARD

Translocation of solutes in plants has been a subject of considerable discussion and research for many years. One phase of the controversy concerns the direction of movement of the elaborate foods and inorganic constituents (1, 2, 3, 4, 5, 6, 7, 9, 10, 11). Can these materials move both upward and downward at the same time in the stem? The purpose of this paper is to show the results of simple experiments which furnish satisfactory proof that backward and forward translocation of solutes occurs regardless of the orientation of the intervening stem tissue between the source of supply and the place where the material is utilized. The methods are not difficult and may be useful to teachers of plant physiology.

METHODS

Two methods were used to cause materials to flow backward through portions of the stems. First, two plants were grafted by the inarch tongue method, and after the union was effected, both tops were removed a short distance above the graft. A few days later one stem, having an active shoot some distance below the graft union, was cut off approximately an inch above the ground level. All shoots excepting one or more at the base of the severed stem were cut off. As the plant grew it formed an *N*-shaped stem (Fig. 1). In some cases several plants were inarched together, the final products being plants with *M*-shaped or *W*-shaped stems, depending upon where the grafts were made. Secondly, a slit was made under a bud and up the stem of a decapitated plant, thus interfering with the normal path of translocation in the stem (Fig. 2). These methods were resorted to after attempts to effect graft unions with inverted scions had failed. Apparently, cells must be properly oriented to meet polar requirements before tissues can grow together. To bring about a complete union, the inverted stem piece had to be turned to an angle of 90° or more. Unions were readily effected if the stems, oriented in the same direction, were grafted parallel or nearly so.

RESULTS

With the methods described above, successful unions and resulting plants were obtained with the following species: *Lycopersicon esculentum* Mill. (tomato), *Bryophyllum pinnatum* Kurz., *Coleus blumei* Benth., *Salvia splendens* Ker., *Nicotiana tabacum* L. (tobacco), *Gossypium hirsutum* L. (cotton), *Dahlia variabilis* Desf., and *Ligustrum ovalifolium* Hassk. (privet). Also tomato scions were grafted on tobacco stocks and vice

versa. In some cases tomato scions were alternated with tobacco, the final stem being the shape of the letter *W*.

As can be seen from Figure 1, materials passing from the soil to the growing tips traveled upward to the graft union, where they crossed over and moved downward to the growing branch, and then again moved upward. To supply the roots with elaborated food, the path, starting from the leaves, was the reverse of that described for the inorganic materials.

These results do not throw new light on the subject of the path of translocation. They do show, however, that the direction of movement is not determined by stem tissue intervening between the distal portions of the

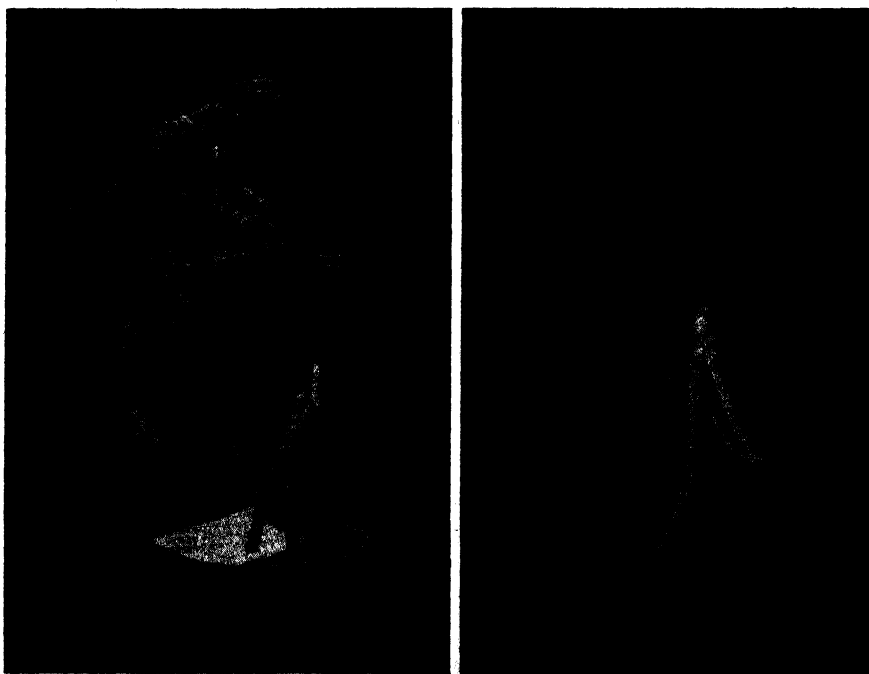


FIGURE 1. Left, tomato, and right, *Bryophyllum*, made by grafting two plants of each species together, after which the tops were cut off and one root system severed. All new shoots were removed as they appeared, excepting those near the base of the scion.

plant. This fact was well demonstrated with split stems of *Coleus* plants as shown in Figure 2. In these experiments incisions were made under young shoots and then the stems were divided upward for a considerable distance. The tops of the plants were removed, leaving only one node above the slit. A few tops were cut off below the node. With the latter treatment, the shoots on the overhanging stem portion wilted readily and made practically no growth. Figure 2 shows the difference in rate of growth in the

two types due to the presence or absence of a node above the slits. The circular insert shows branching of vascular bundles at a node, thereby affording a natural bridge for lateral translocation of materials in *Coleus*. To reach the growing points, inorganic elements traveled up one side of the stem, crossed over, and then down the other side. Materials elaborated in the leaves traveled in the opposite direction to reach the roots. There was occurring at the same time both upward and downward translocation in

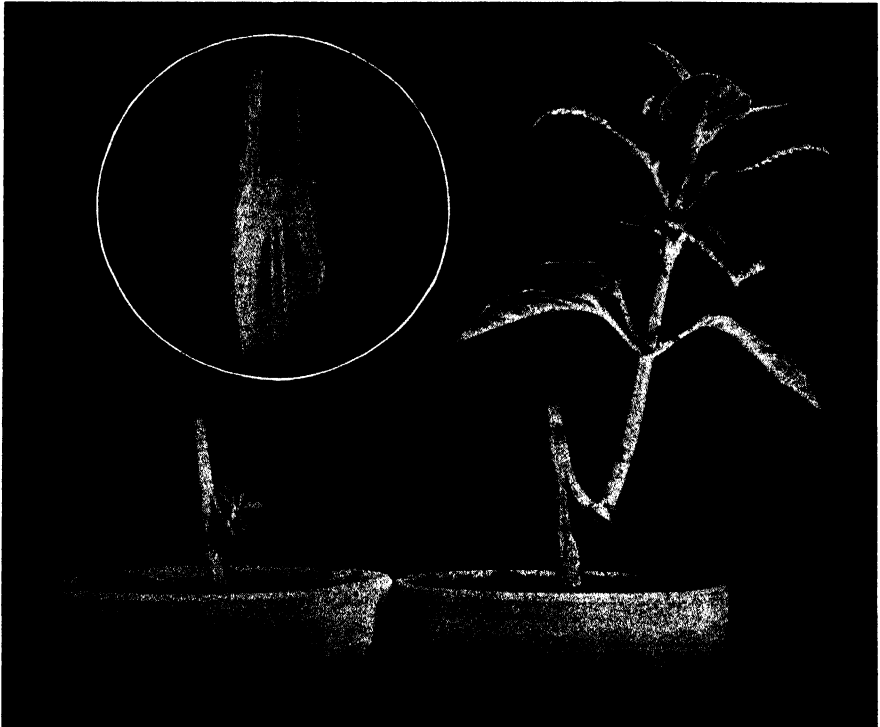


FIGURE 2. Two *Coleus* plants with shoots, originally of the same size, growing on overhanging portions of split stems. Left, top of the plant cut off below a node; right, top cut off above a node; circular insert, longitudinal section ($\times 2$) of the stem portion above the slit of plant on right, to show branching of vascular bundles at a node.

the stems. The cells along the way apparently did not control the direction of movement, but provided the paths through which materials traveled.

The poor growth of *Coleus* shoots where the stems were cut off below a node (Fig. 2) was due to the peculiar morphology of vascular bundles. Dissected and sectioned stems showed that branching of the bundles occurred at the nodes, thus affording a cross-over path for translocation (Fig. 2). When stems were cut off below a node the natural bridges were eliminated and materials could not be readily transferred. Caldwell (1,

p. 37) gave a good description of the vascular anatomy of *Coleus* stems in his paper on movement of food materials in plants.

Plants grafted by the inarch method made a bridge at the graft union which served as a path for material translocation. When the grafted stocks were severed near the ground level and placed in eosin solution, the red

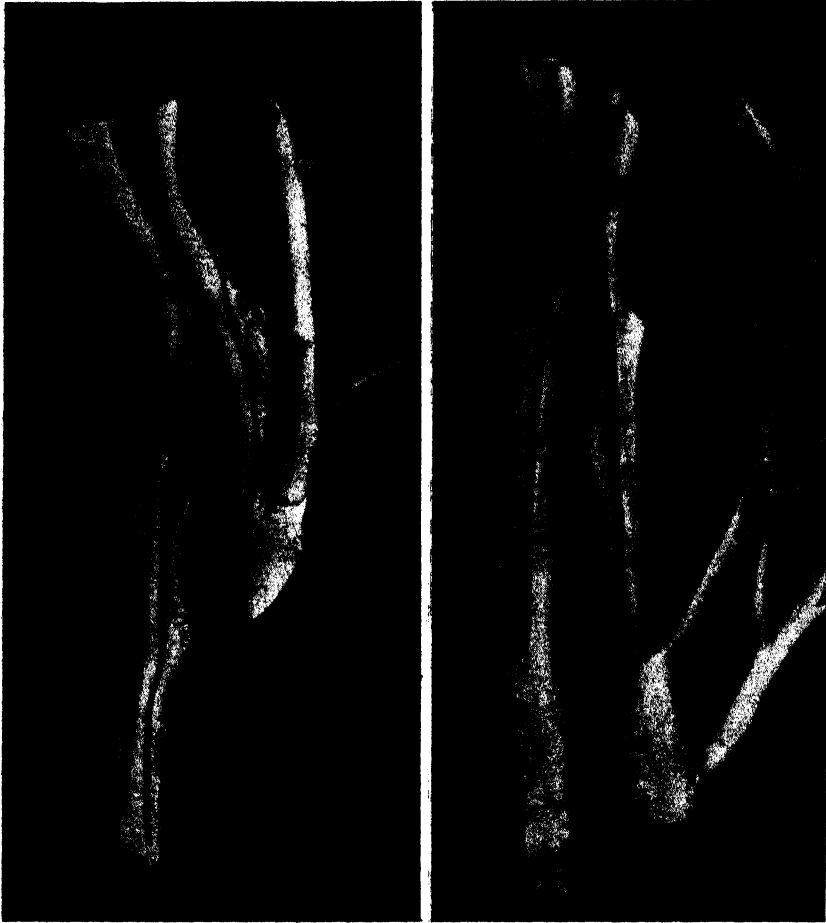


FIGURE 3. Stems of grafted tomato plants with black lines showing, diagrammatically, the movement of eosin after root system was severed and the cut end of the stem placed in a weak solution of the dye. Left, stem split to expose the pith; right, bark removed down to the xylem.

dye moved readily into the growing shoots of the scions (Fig. 3). How the new tissues, which formed at the unions, were joined to make continuous vascular bundles is not known.

DISCUSSION

The ideas involved in these experiments are not entirely new, though the methods may be. Hales (8) described three apple trees joined by inarching, of which the middle tree flourished although its roots were dug out of the ground and suspended in the air. Strasburger (12, p. 936) told of cutting off a branch of red beech which had grown to another one above and found that it thrived like the other branches. Strasburger (12) also described an experiment of Duhamel in which two elm trees were grafted together after which one of them was cut off from its roots. The cut tree bore leaves below the graft union. Swingle, Robinson, and May (13) in discussing the advantages of a "nurse-graft, Y-cutting" state that water containing dissolved mineral salts will, if necessary, move backward through the wood of leafy branches, but that carbohydrates and nitrogenous reserve foods move downward in the phloem elements and do not move or at least move with great difficulty in the reverse direction. This may be true for growing plants under normal conditions. In our experiments, however, elaborated foods as well as salts, appeared to move upward and downward through the stems. When plants are growing there is a continual need for mineral salts which move to the place where they are utilized. Similarly, the growing roots require food elaborated in the leaves. The orientation of the intervening stems is of no consequence, activity at some distant point being the determining factor. This view is not out of accord with the conclusions of Curtis (5) and Mason and Maskell (10, 11) that the living cells take an active part in translocation of materials, for protoplasmic streaming should have the same effect whether the direction of material flow be backward or forward.

SUMMARY

Plants were grafted by the inarch method and later pruned so as to produce plants with abnormally oriented stems, causing food materials and salts to be translocated upward and downward at the same time.

Shoots were caused to grow on overhanging portions of split stems. To reach the growing points solutes were forced to travel upward in one part of the stem and downward in the other part.

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SEA-WATER TOLERANCE OF VALLISNERIA SPIRALIS L. AND POTAMOGETON FOLIOSUS RAF.

W. S. BOURN

INTRODUCTION

In a previous paper (2) the results of experiments on the sea-water tolerance of five species of aquatic angiosperms were reported. Among these plants was included *Vallisneria spiralis* L. (wild celery). Those experiments, however, were carried on from January 20 to March 20 with "winter buds" of *Vallisneria*. Since these buds are rather dormant in fresh-water cultures grown in the greenhouse during the winter and make little growth in the early part of the season, it was thought best to repeat the experiments at a time when the buds were no longer dormant and the plants were capable of a more active growth. The experiments were repeated from May 22 to July 20.

At the same time experiments were carried on for the purpose of determining the concentration of sea water which *Potamogeton foliosus* Raf. (leafy pondweed) can tolerate. This plant has been reported by Metcalf (7) as being able to withstand a high concentration of salts (higher concentrations than many other pondweeds are known to withstand) in the alkaline lakes of North Dakota. *P. foliosus* is completely dormant in greenhouse cultures during the winter months and under favorable conditions makes no appreciable growth until after March 1.

Both *Vallisneria spiralis* L. and *Potamogeton foliosus* Raf. are widely distributed in waters throughout the United States and are among our most important food plants for migratory wild fowl (4, 5, 6, 7). Since much of the aquatic area remaining in this country as feeding grounds for migratory game birds, such as wild ducks and geese, is situated along the Atlantic Coast where considerable portions contain various amounts of sea water, it is of great interest to determine under controlled conditions the amount of salt water which each species of duck-food plants can withstand. The results of such determinations should be of special interest to conservationists and sportsmen planning to establish aquatic areas as refuges or attractions for migratory water fowl.

EXPERIMENTAL METHODS

The experiments were performed out-of-doors in stoneware vessels of approximately 45 liters capacity. Ten kilograms of good garden soil were added to each vessel. The vessels were then arranged in two series of eleven each and the various 'dilutions of sea water added. Sea water from Long Island Sound was used in the preparation of the solutions. This water,

which was found, according to the method described by Denny (3) for determining the saltness of brackish water, to be approximately 80 per cent normal sea water (3.5 per cent total salts is considered normal sea water), was diluted with tap water to make the following concentrations of sea water for each species in separate cultures: 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, and 36 per cent. The solutions thus made up were renewed in each culture vessel every second day, except when rainfall made necessary more frequent changes. The temperature of the culture solutions varied during the experiment from 19° to 24° C. In order to maintain the temperature as low as possible the solutions were changed often and the cultures were protected from intense sunlight with slat shades (laths one inch in width and spaced one inch apart on a frame). Concentrations of dissolved carbon dioxide and oxygen of approximately 60 and 8 parts per million by weight, respectively, were maintained fairly constant in each culture throughout the experiment. This was accomplished by bubbling the gases through Berkfeld filter cores into the solutions and checking the concentrations by the methods outlined by the American Public Health Association (1). It was found unnecessary, however, to add oxygen to the growing cultures soon after the beginning of the experiments, as the concentration of the gas in the more rapidly growing cultures exceeded 8 parts per million.

The plant material used in these experiments was taken from cultures grown for several years in the greenhouse. The stock was collected originally from the waters by Back Bay, Virginia, which at the time was slightly brackish (2), but for several years the plants had been cultured in fresh water in the greenhouse. In the case of *Vallisneria spiralis* L., ten "winter buds," of about the same size and weight, which had just begun to produce roots and leaves, were used in each culture. With *Potamogeton foliosus* Raf., ten terminal, unbranched cuttings, each 15 cm. in length and approximately equal in weight, were employed in each culture. These cuttings were allowed to root in fresh water before being transferred to the salt water solutions at the beginning of the experiments. After eight weeks the experiments were discontinued. The plants were carefully removed from the cultures, washed several times, and brought to a constant dry weight in an oven at 60° C. The percentage increase in dry weight in each culture was then calculated by comparing with the dry weight of comparable samples taken at the beginning of the experiments.

RESULTS

The effect of different concentrations of sea water on the growth of *Vallisneria spiralis* L. and *Potamogeton foliosus* Raf. under favorable conditions of soil, temperature, light, and carbon dioxide and oxygen supplies is shown in Figure 1. The results are presented in percentage increase in dry weight, using as a basis of calculation the percentage dry weights of

comparable samples taken at the beginning of the experiments.

While increasing concentrations up to 8 per cent sea water stimulated the growth of *Vallisneria spiralis* L., the percentage increase in dry weight in any concentration of sea water used over that of the check plants grown in fresh water was only very slight. The leaves of the plants grown in cultures containing 20 per cent sea water were small, slender, and somewhat etiolated at the end of the experiments. This concentration marks the upper limit of the amount of sea water which the *Vallisneria* plants used in

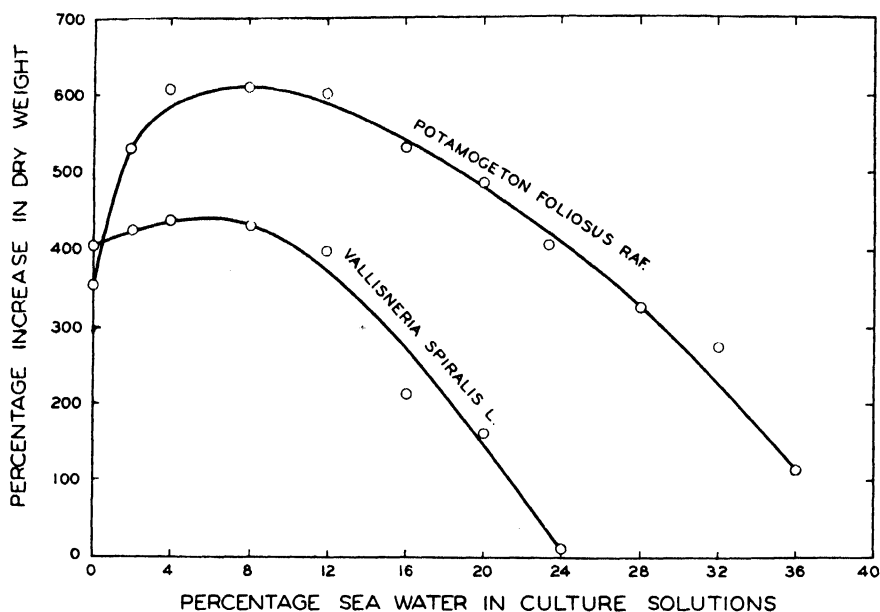


FIGURE 1. Growth of *Vallisneria spiralis* L. and *Potamogeton foliosus* Raf. in different concentrations of sea water.

these experiments tolerated. The plants in the cultures containing 24 per cent sea water made a very insignificant growth and were dead and disintegrating at the conclusion of the experiments. In the cultures containing 28, 32, and 36 per cent sea water, respectively, the "winter buds" completely decayed soon after the initiation of the experiments. The results confirm those of earlier work on this plant (2).

Concentrations up to 24 per cent sea water stimulated the growth of *Potamogeton foliosus* Raf. The results indicate, however, that the optimum salt content for the promotion of growth in this plant lies between 4 and 12 per cent sea water. In common with other *Potamogetons* tested (2), *P. foliosus* was found to tolerate a much higher concentration of sea water than *Vallisneria spiralis* L. In fact, *P. foliosus* withstood a concentration

of 36 per cent sea water, although the plants in the culture containing that salt content at the end of the experiment were small, spindling, unbranched, and had produced no seeds. The plants in cultures containing a salt content of 24 per cent and less sea water were branched profusely and produced an abundance of flowers and seeds. From these results it can be concluded that *P. foliosus* cannot tolerate a much higher concentration than 36 per cent sea water.

DISCUSSION

The present results indicate that *Vallisneria spiralis* L. is strictly a fresh-water species. Although this plant has been reported as growing in parts of Back Bay, Virginia, and Currituck Sound, North Carolina, in water known to contain over a period of years variable percentages of sea water, the average salt content of these waters during that time did not exceed 10 per cent normal sea water (2). Furthermore, the writer in July, 1926, observed stunted specimens of this plant growing in the eastern part of Albemarle Sound, North Carolina, where the salt content of the water at the particular time was equivalent to 14 per cent sea water. In any of these cases, however, where *Vallisneria* was found to grow, the salt content of the water did not exceed the highest concentration of sea water tolerated by the plant in the present experiments. To such conditions of salinity as occur in the eastern part of Albemarle Sound, which at all times must contain at least a small percentage of sea water, *Vallisneria* may have become adapted after a long period of time, especially since the plant grows abundantly in adjacent and connected fresh waters. Such a conclusion, however, cannot be drawn for all bodies of salt water, for example, the alkaline lakes in the interior of this country, of which the different salts do not occur in the same proportions as those found in sea water. Metcalf (7), in a study of the aquatic flora and the concentration of salts in 72 alkaline lakes of North Dakota, found such aquatic angiosperms as *Potamogeton pectinatus* L. growing abundantly in waters with total concentrations of salt ranging from 350 parts per million (Ranch Lake), or a content equivalent to one per cent sea water, to 35,873 parts per million (Swamp Lake), a total salt content greater than that of normal sea water. He did not, however, find *Vallisneria spiralis* L. growing in any of the 72 lakes. He remarks (p. 4) that one of the interesting features of the aquatic flora in the numerous lakes studied by him was the absence of wild celery, although the plant was present in abundance in the fresh-water lakes of adjoining Minnesota. McAtee (6) studied the duck-food plants growing in 44 alkaline lakes in the sandhill region of Nebraska. Although *Vallisneria spiralis* L. and *Potamogeton pectinatus* L. are frequently associates, he found *Potamogeton pectinatus* growing in all the lakes except one (Trout Lake), but he found no *Vallisneria spiralis* in any of them. He states (p. 38) that the most notable deficiency in the list of plants observed was wild

celery (*Vallisneria spiralis*) and expresses the opinion that this plant undoubtedly will grow in practically all the lakes of the sandhill region, and, furthermore, that this plant probably will grow anywhere that sago pondweed (*Potamogeton pectinatus*) does. Pool (8), in a study of the vegetation of this same region, reported (p. 275) that the waters of practically all of the lakes "contain considerable quantities of saline and alkaline compounds." He gives the total concentration of dissolved solids, including organic matter, in the water of a few of the lakes, which ranges from 0.05 per cent in Hackberry Lake to 0.47 per cent in Little Alkali Lake. It will be seen readily that such total concentrations of salts, including organic matter, scarcely exceed the concentration of sea water, 12 per cent (0.42 per cent total salt), tolerated by *Vallisneria spiralis* L. in the present experiments. From these observations and from the results presented in this paper it seems reasonable to conclude that *Vallisneria* is more properly a fresh-water plant and does not tolerate the amount of salt which a plant such as *Potamogeton pectinatus* L. is able to withstand. The fact that this plant is not found growing in saline or alkaline lakes appears especially significant when it is considered that it is subject to the same natural agencies of dissemination as other aquatic angiosperms. Until more experimental evidence is presented to the contrary, this plant is not recommended for transplanting from fresh water to any water containing appreciable amounts of salts.

Confirming the observations of Metcalf (7), the results of the experiments here reported show that *Potamogeton foliosus* Raf. will withstand a relatively high total concentration of salts. Metcalf found this plant growing abundantly in the alkaline lakes of North Dakota which contained a total salt content as high as 2,512 parts per million (0.25 per cent total salts). In the present experiments the results show that this plant can tolerate a concentration of 36 per cent sea water, or a total salt content of 1.26 per cent, although the optimum concentration lies between a total concentration of 0.28 and 0.42 per cent. In nature this plant seems to be limited to rather shallow water, but in greenhouse cultures it has been grown successfully in tanks six feet in depth. Like *Potamogeton pectinatus* L. (sago pondweed), *Potamogeton foliosus* Raf. (leafy pondweed) can be recommended for transplanting either in fresh or brackish waters.

SUMMARY

The sea-water tolerance of two species of aquatic angiosperms, *Vallisneria spiralis* L. and *Potamogeton foliosus* Raf., two important duck-food plants, was determined, using stock plants which had been growing in fresh-water cultures in the greenhouse for several years. *Vallisneria spiralis* could not be grown successfully in concentrations of sea water greater than 12 per cent, or in a total concentration of salts greater than

0.42 per cent. *Potamogeton foliosus* withstood a concentration of sea water as high as 36 per cent, or a total concentration of salts equal to 1.25 per cent.

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SEPARATION OF CELLULOSE PARTICLES IN MEMBRANES OF COTTON FIBERS BY TREATMENT WITH HYDRO- CHLORIC ACID

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The cellulose nature of the ellipsoid particles which enter into the formation of plant cell membranes has been indicated by their refractive indices, their double refraction, and their reaction to the H₂SO₄-iodine test (1). There remained a fourth important method of analysis—that of X-ray diffraction. For this purpose it was necessary to obtain a relatively large mass of cellulose particles from which the non-cellulose membrane constituents had been removed.

In the earlier report (1) a microchemical method of removing the non-doubly refractive cementing substances from the cell walls was described. The particles separated by means of mild pectic solvents were apparently unaltered by the treatment and furnished excellent material for study of their individual properties. The small fragments of membranes involved in such a procedure yield, however, a relatively small number of particles. In applying this method on a macrochemical scale for the purpose of securing larger quantities of cellulose particles, the resulting mass of material has not been entirely free of the non-cellulose substances. Among the various methods used in an attempt to remove completely the cementing materials, treatment with HCl, according to the procedure reported here, has proved to be most useful.

METHOD

One gram of seed cotton (*Gossypium hirsutum* L.) and 25 cc. of HCl (sp. gr. 1.19) were placed in each of a series of six 100 cc. Pyrex test tubes. The tubes were kept at room temperature and at intervals of $\frac{1}{2}$ hour, 1 $\frac{1}{2}$ hours, 2 $\frac{1}{2}$ hours, 3 $\frac{1}{2}$ hours, 18 hours, and 5 days, one tube was removed from the series, centrifuged, and the contents thoroughly washed with distilled water.

EFFECT OF TREATMENT WITH HCl

Examination of the residues from the $\frac{1}{2}$ -hour sample showed that the original limiting membrane of the fiber (1) had been almost completely removed by the action of the acid and that the wall structure had become much less compact. In a few areas the structure of the fibrils was clearly visible, showing that separation of the particles had already begun. In the

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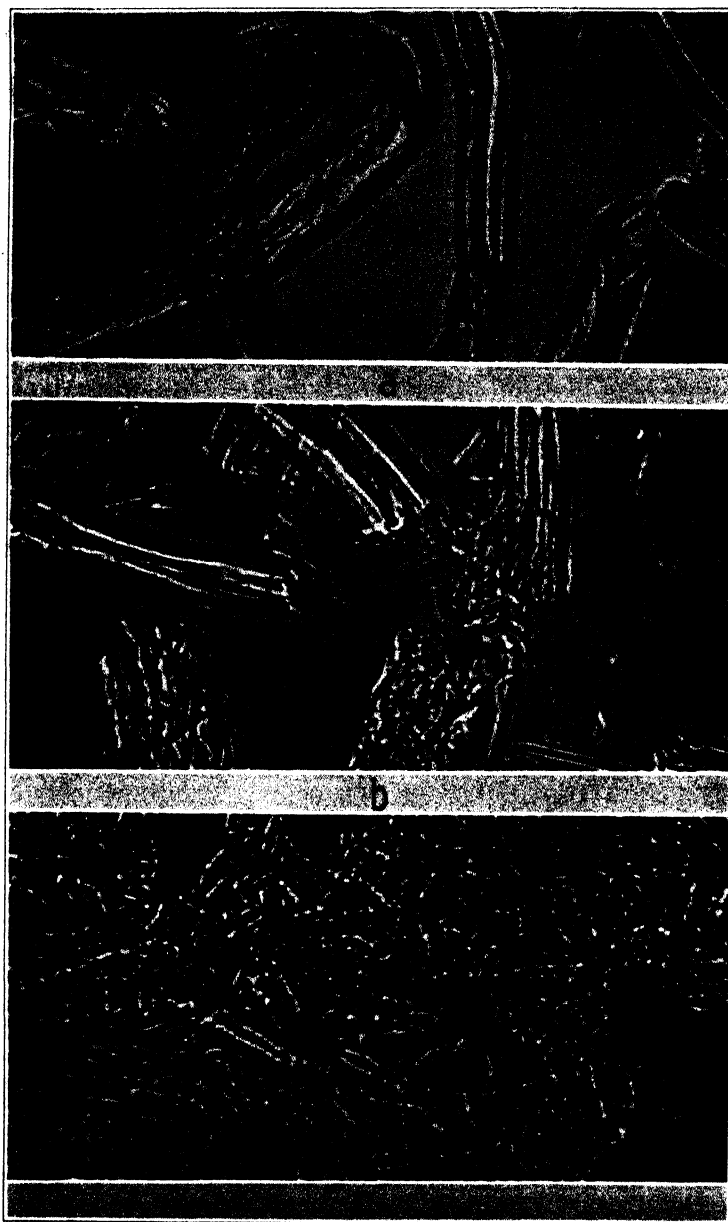


FIGURE 1, (a) Portions of fibers from the residues after treatment for $2\frac{1}{2}$ hours with HCl. The fibril and particle structure of the membranes are shown as well as the cross-sectional rupturing of the fiber. $\times 350$. (b) More advanced degree of fiber dissociation after 18 hours' treatment with HCl. $\times 350$. (c) Extreme degree of fiber dissociation after 5 days' treatment with HCl. $\times 350$.

1½-hour sample the outer non-cellulose membrane was entirely removed, the fibers were swollen, and the fibril and particle structure were much more evident. A few instances of lateral rupturing of the fibers, so conspicuous in the 2½- and 3½-hour samples, could be found. This cross-sectional cleavage is illustrated in the 2½-hour sample shown in Figure 1*a*. As a result of such breaking the 3½-hour sample consisted entirely of short lengths of fibers. The 18-hour sample illustrated in Figure 1*b* was made up of these short pieces of fiber in various stages of separation into fibrils and particles. The 5-day sample contained the relative proportions of particles, fibrils, and wall-fragments shown in Figure 1*c*. Slight pressure upon the cover glass of both the 18-hour and 5-day mounts destroyed all traces of the previous arrangement of the particles in the fiber wall, indicating that the cementing substances had been removed. No pectic substance remained as shown by microchemical tests. Figure 2*a* and *c* show smaller masses of these separated particles at higher magnifications and illustrate more clearly the unchanged proportions of the particle. The long axes of these isolated particles may assume any position with respect to the surface of the slide from the parallel to the perpendicular. This accounts for the spherical appearance of some of the particles in the illustrations. These same particles may be turned into any desired position by properly directed pressure upon the cover glass.

In another series, different concentrations of HCl (sp. gr. 1.19) were used in treating similar samples of cotton at room temperature for a period of 8 days. The 10 per cent sample showed slight swelling of the fibers, but convolutions were still present in large numbers of both the thick-walled and thin-walled fibers. In the 25 per cent sample the fibers were swollen to a greater degree and only those with thick walls retained convolutions. The 40 per cent sample clearly indicated the fibril structure of the wall. The 60 per cent sample showed both the fibril and particle structure and furnished excellent examples of cross-sectional rupturing. The 80 per cent sample had reached a state of dissociation comparable to the 18-hour treatment in undiluted HCl.

The fact that unaltered particles in large quantities were obtained in the residues does not obviate the possibility that hydrolysis of some of the cellulose particles may have occurred during the treatment.

PROPERTIES OF THE SEPARATED PARTICLES

The H₂SO₄-iodine test applied to mounts of separated particles produced a swelling accompanied by the characteristic blue coloration. After the treatment with HCl the particles are more sensitive to the swelling action of H₂SO₄, passing over quickly into a blue colloidal mass. It is necessary, therefore, to use care in applying the H₂SO₄ in order to prevent loss of the original contour of the particle (Fig. 2*b*).

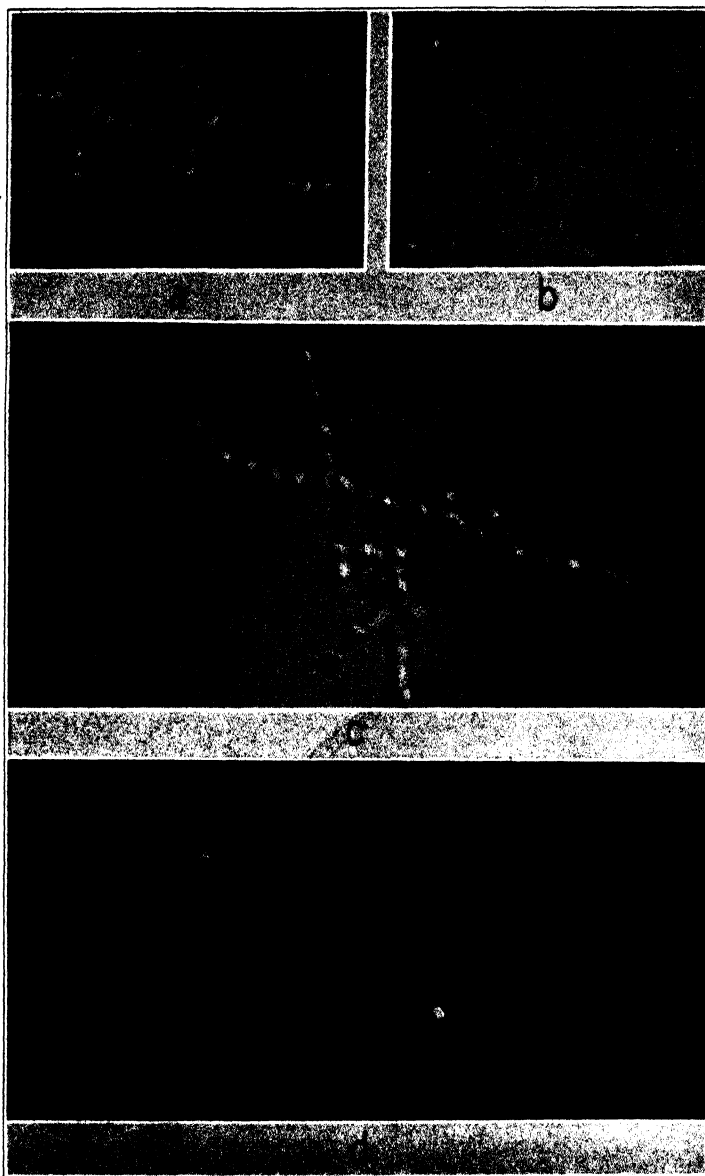


FIGURE 2, (a) A small mass of separated particles with long axes at angles ranging from parallel to perpendicular, with respect to the surface of the slide. $\times 1380$. (b) Separated particles after treatment with H_2SO_4 and iodine. The amount of swelling is indicated by comparison with Figure 2a. The dark area around the edge of each particle shows the position of the dark-blue, partially dissolved cellulose. $\times 1380$. (c) A mass of separate particles at higher magnification. $\times 1630$. (d) Two cellulose particles oriented at 45° to the direction of a beam of polarized light and at right angles with respect to their own long axes. $\times 1380$.

In polarized light the particles were clearly doubly refractive. When oriented with their long axes at 45° with respect to the direction of the beam, they were in the positions of greatest brightness (Fig. 2*d*); when placed with their long axes parallel to the beam, they were extinct.

X-ray analysis of a small pellet of the particles gave the typical Debye-Sherrer cellulose diffraction pattern. A similar pellet, when subjected to mercerization treatment produced a diffraction pattern of mercerized cellulose. These diffraction patterns are described and illustrated in a separate paper (2). The results of this method of analysis are of importance in contributing to the earlier evidence as to the cellulose nature of the particle as well as in indicating the unaltered condition of the particles in the residues from HCl-treated membranes.

SUMMARY

The removal of non-cellulose substances from the cellulose particles in the living cytoplasm and in the mature cell wall of the cotton fiber was accomplished previously by means of mild pectic solvents.

In applying this method on a macrochemical scale for the purpose of securing larger quantities of cellulose particles the resulting mass of material was not entirely freed from the non-cellulose substances.

Treatment of mature cotton fibers with HCl (sp. gr. 1.19) for periods ranging from one-half hour to five days resulted in the removal, during the longer intervals, of the non-cellulose constituents. Large quantities of cellulose in the form of particles were obtained for experimental purposes.

Microchemical, optical, and X-ray diffraction analyses showed that the essential cellulose nature of the particles was unaltered.

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X-RAY DIFFRACTION PATTERNS OF CELLULOSE PARTICLES AND INTERPRETATIONS OF CELLULOSE DIFFRACTION DATA

WANDA K. FARR¹ AND WAYNE A. SISSON²

The recent description of the formation of cellulose membranes by microscopic particles of uniform size in linear arrangement by Farr and Eckerson (5) was based upon microscopic and microchemical technique applied to growing cells and to mature cell walls. The various methods used in both ordinary and plane-polarized light made possible the determination, in the young cell, of the ellipsoid shape of the particle, its approximate size ($1.5 \times 1.1\mu$), its anisotropic behavior, the arrangement of its long axis parallel to the main axis of the fibril, and the thin coating of pectic substance upon its surface. Mature cell membranes were separated into fibrils and these, in turn, into particles by means of suitable pectic solvents. The size and optical properties of these particles from mature membranes established their identity with those observed during cell wall formation.

The cellulose nature of the particles was determined by their double refraction, their reaction to the H_2SO_4 -iodine test, and their refractive indices. The technique in these three instances required small amounts of material. In order to free larger quantities of the particles from the non-cellulose substances which surround them, the fibers were treated with HCl (sp. gr. 1.19) for 18 hours, centrifuged, and washed. The residues consisted of cellulose particles whose appearance, optical properties, and chemical reactions indicated that they had not been affected by the treatment (6).

X-RAY DIFFRACTION PATTERNS OF CELLULOSE PARTICLES

These separated particles, when pressed into a pellet suitable for mounting upon the X-ray diffraction apparatus, give a typical Debye-Sherrer pattern of cellulose as shown in Figure 1*a*. Figure 1*b* is a powder diagram of cellulose obtained by cutting cotton fibers into small fragments and pressing them into a pellet. The number of diffraction lines, their distance from the central beam, and the general characteristics of the lines themselves are seen to be strikingly similar in both patterns. Careful measurement serves to establish the cellulose nature of the separated particles

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by comparison with the pattern of the known cellulose sample. Figure 1c, from a bundle of paralleled cotton fibers is seen to differ from *a* and *b* only in the degree of regularity in orientation of the cellulose. Further evidence of its intact condition is obtained from a diffraction pattern of

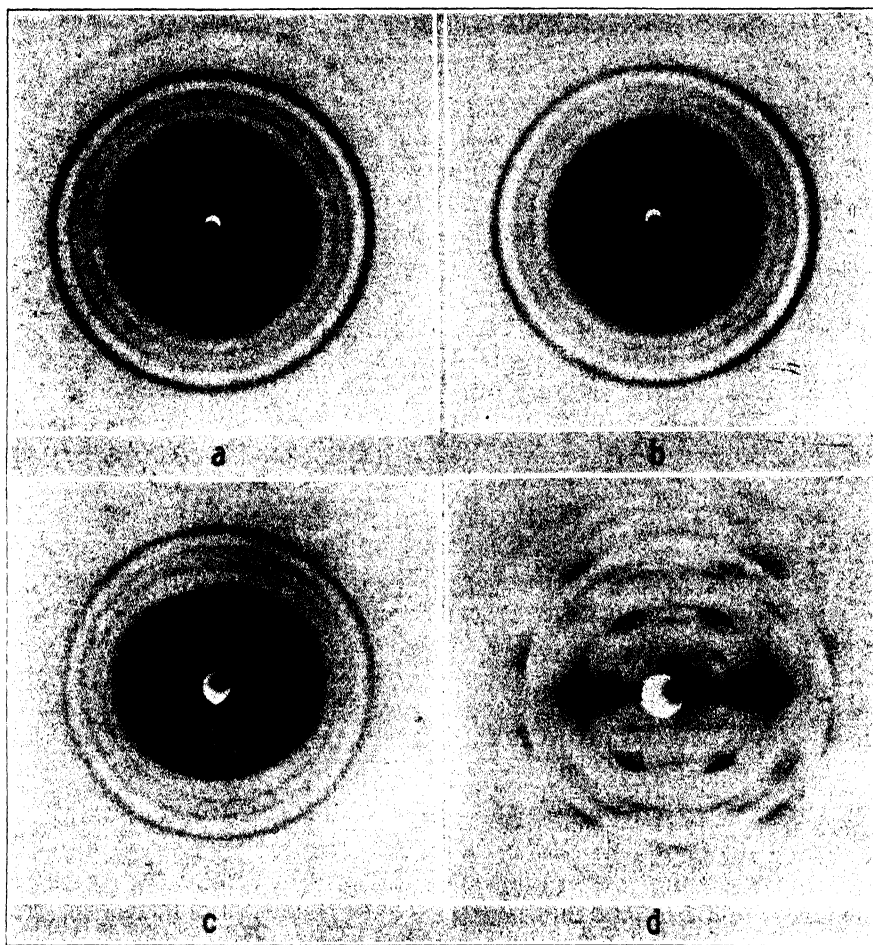


FIGURE 1. X-ray diffraction patterns of cellulose. (a) Cellulose particles from cotton fibers separated by 18-hour treatment with HCl; (b) pulverized cotton fibers; (c) paralleled cotton fibers; (d) paralleled ramie fibers.

a mass of separated particles which had been mercerized by treatment with 18 per cent NaOH. The pattern is typical of mercerized cellulose.

The fact that cellulose gives definite X-ray diffraction lines is, in itself, proof that cellulose is in most cases crystalline (1, 3). Since a single fiber gives a Debye-Sherrer or fiber pattern instead of a Laue pattern (4), a

discontinuous instead of a continuous structure of crystalline cellulose is implied.

INTERPRETATIONS OF X-RAY DIFFRACTION DATA

In the interpretation of X-ray diffraction patterns of cellulose, there are several *characteristics* of the pattern which must be accounted for on the basis of the fine structure of the fiber:

1. Number of diffraction rings or spots (monochromatic pinhole method).
2. Distance of each from the central beam (related to the spacings of the corresponding planes by the equation $n\lambda = 2d \sin \theta$).
3. Relative density of the diffraction lines.
4. Concentration of density into localized intensity maxima (spots) around each ring.
5. Width or diffuseness of the diffraction lines.

It is now generally accepted that the fundamental unit of cellulose structure is the ring-formed β -glucose residue and that these residues are connected by glucosidic oxygen bridges in the one-four positions to form long molecular primary valence chains in which the glucose rings are alternatively faced in opposite directions to form the unit cell. With our knowledge of the chemistry of sugars and polysaccharides and the size and crystal habits of carbon, hydrogen, and oxygen in other organic crystals, we may feel fairly confident that the number of X-ray lines or rotation spots, their corresponding spacings, and relative intensities (*characteristics* 1, 2, and 3) may be satisfactorily explained by the definite assigned positions of the glucose residues in the unit cell (12). This unit cell is extended or repeated in all directions to build up a crystalline aggregate.

Characteristic 4 gives a clear picture of the way in which these cellulose chain aggregates are arranged with reference to the fiber axis, but as to their exact size and nature the information obtained from *characteristic 5* is less definite. Although the X-ray data seem to be satisfactorily explained on the assumption that these crystalline aggregates are submicroscopic in size, are much longer than they are thick, and are arranged with their long axes roughly parallel or spiral with respect to the main axis of the fiber, there is no universal agreement as to their exact nature. In the present discussion they will be referred to as "crystallites." In the absence of any other known structural unit between the *cellulose unit cell* and the *fibril*, they are commonly associated with Nägeli's *micellae*.

Orientation of crystallites. The indication of a definite orientation of the cellulose chains in ramie (*Boehmeria nivea* L.) in such a way that the *b* axes of the unit cells are approximately parallel to the fiber axis is shown

in Figure 1d. The cellulose diffraction lines are concentrated into small arcs. If it is assumed that the cellulose chains are parallel to the long axis of the cellulose particle, the conception of particles arranged end to end to build up the fibril would account for this phase of the diffraction phenomena. A more complete discussion of crystallite orientation in cellulose fibers is given elsewhere (14).

Size of the crystallites. It is well known from work on inorganic crystals that if a single crystal is ground into smaller and smaller crystalline fragments, the Laue diffraction spots become more and more numerous with progressive decrease in size, until an average crystal diameter of between 1×10^{-5} and 1×10^{-4} cm. is reached. At this point the numerous Laue spots begin to merge into smooth, uniform, Debye-Sherrer rings which remain constant between the approximate limits of 1×10^{-4} and 1×10^{-6} cm. The diameter of the cellulose particle as estimated from microscopic observations is approximately 1.1μ or 1.1×10^{-4} cm. This is well within the range of crystal size necessary to give the powder pattern obtained from the separated cellulose particles. Debye-Sherrer rings obtained from a sample of silica (3, p. 349) having an average particle size of 4.4×10^{-4} cm. or four times larger than the cellulose particles may be cited in this connection.

Width of diffraction lines. In well oriented cellulose fiber diagrams (Fig. 1d) where the crystallites have a common axis parallel to the fiber axis, the X-ray pattern approaches a single crystal rotation photograph. The width of the lines arising from the planes parallel to the b axis of the crystallite (equator lines) is greater than that from the planes perpendicular to the b axis (meridian lines). This difference in line breadth is usually attributed to the existence of rod-like crystalline micellae. The interpretation is based upon the fact that, in other crystalline substances, when the average crystal size becomes less than about 1×10^{-6} cm., the diffraction lines become broader and more diffuse due to the lack of sufficient diffracting planes. It is possible from the degree of this broadening to estimate the crystallite size within the colloidal range of 1×10^{-6} to 1×10^{-8} cm. Upon this basis Hengstenberg and Mark (7) have estimated the micellae in ramie to be approximately 5×10^{-7} cm. in diameter and 5×10^{-6} cm. in length ($50 \times 500 \text{ \AA}$).

As pointed out by Hengstenberg and Mark, the equation employed for calculating the micellar dimensions from this type of diffraction data is only valid provided the following assumptions are fulfilled:

1. Absorption of X-rays by the sample is negligible.
2. Micellae have only one definite size.
3. All micellae have identical crystal structure.
4. Heat motion has no appreciable effect.
5. The lattice structure of each micella is perfectly formed (i.e., not deformed by the presence of impurities, pressure, or tension).

For many inorganic crystals it may be safely assumed that all of these conditions are fulfilled. When dealing with the complicated, less familiar structure of cellulose, however, the possibility that one or more of these factors may have an effect upon line breadth cannot be overlooked. Since many non-cellulose materials are so closely associated with cellulose during its formation in the cell wall, they may produce a strained or distorted lattice. It is even possible that they may give a diffraction pattern which is superimposed upon the cellulose pattern and alters its appearance. In this connection one of the authors (14) has observed that if the cellulose from certain fibers, which originally gave broad, diffuse, diffraction rings, is separated from the non-cellulose materials, an X-ray pattern is obtained which approaches that of cotton cellulose. It would seem probable that the diffuseness of the lines from the untreated fibers was due to the presence of non-cellulose materials rather than that an increase in micellar size had taken place during their removal. Herzog (8) has advanced the theory that tension during growth causes the orientation present in natural fibers. Farr and Eckerson (5) have observed that when cotton fibers are treated with solvents of the non-cellulose cementing substances, the fibrils, which have been in the form of a spiral, immediately straighten out. This would indicate that the fibrils had been in a state of tension in the fiber wall. If the broadening of the diffraction lines is due either to impurities or to strain, the question may arise as to why the equator and meridian lines are not equally affected. It will be remembered in this connection that the substitution of the hydroxyl groups for other groups often affects only the planes parallel to the cellulose chains, the identity period remaining constant (11).

Swelling phenomena. It has been suggested repeatedly that there is additional evidence for the micellar hypothesis in the swelling phenomena of cellulose membranes. There is no change in the X-ray pattern produced by swelling and subsequent stretching except in orientation (9). These data, however, do not necessarily indicate the existence of micellae. It would seem to be equally logical, now that a definite microscopic cellulose particle has been demonstrated, to assume that the particle is the aggregate which moves as a whole during swelling and stretching and that the diffuse X-ray lines are due to a lack of perfect crystal structure within the cellulose particle.

There is a growing feeling among research workers that the micellar hypothesis does not adequately explain the behavior of native cellulose fibers. Several investigators have recently suggested that a more or less continuous structure, in which the cellulose chains have crystallized into areas of perfect and imperfect crystallinity, explains the known facts concerning the fiber better than the micellar hypothesis. Sponsler (15) has suggested that a meshwork structure consisting of bent and warped cellu-

lose chains is in keeping with his theories of the mechanism of cell wall growth. Neale (13, p. 233) finds no necessity for assuming the existence of well defined micellae in order to explain the chemical behavior of cotton fibers and as an alternative explanation for the X-ray data has suggested "a rythmic variation from a perfectly crystalline structure without sharp discontinuities." Astbury (2, p. 204) has suggested that instead of the pre-existence of sharply defined micellae, "rather does the available evidence point to a self contained system riddled through and through by crystallization 'faults'."

In conclusion it may be suggested that the conception of cell membranes composed of crystalline particles surrounded by non-crystalline substances consisting largely of pectic material holds a unique position with respect to the older discontinuous micellar theory and the more recent continuous-structure theory. The essential features of the micellar theory are the existence of rod-like submicroscopic crystalline masses oriented with respect to the fiber axis and separated from one another by a layer of amorphous material which allows the micellae to move as a unit during intermicellar swelling (9) and plastic flow (10). With the exception of shape and size these attributes are analogous to those of the cellulose particle. If the imperfect crystalline formations of the continuous structure theory are imperfect only to the extent necessary to produce broad diffraction lines, the details of the recently demonstrated membrane structure (5) would agree in this instance as well.

Knowledge of the existence of the cellulose particle and its relationship to the non-cellulose materials in the plant cell membrane raises the question of the necessity for the use of the micellar hypothesis in the interpretation of X-ray diffraction data. It must be emphasized at this point that the possibility of a further subdivision of the cellulose particle into smaller, possibly submicroscopic, crystalline masses is not obviated by the new conception of membrane structure. Final interpretations, however, must await further experimentation in all phases of cellulose analysis.

SUMMARY

Cellulose particles, obtained by treating cotton fibers with hydrochloric acid to remove the non-cellulose cementing materials, when pressed into pellets and mounted upon an X-ray diffraction apparatus, gave a typical Debye-Sherrer cellulose pattern. When the particles were treated with 18 per cent NaOH a pattern characteristic of mercerized cellulose was obtained.

Certain X-ray diffraction phenomena which heretofore have seemed to necessitate the assumption of the existence of hypothetical submicroscopic micellae are explained equally well by these microscopically visible particles of cellulose, observable as units in the young cotton fibers but united

in the later stages of growth by non-cellulose cementing substances to form fibrils in which the cellulose particles can no longer be seen as individuals.

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AFTER-RIPENING AND GERMINATION OF COTONEASTER SEEDS

JOHANNA GIERSBACH

INTRODUCTION

The germination of the seeds of the genus *Cotoneaster* offer great difficulty to practical growers and to scientists. There is need of a careful scientific study of the germination behavior of these seeds with the hope of developing effective practical methods of seedling production. Pammel and King (10) planted seeds of *Cotoneaster acutifolia* Turcz. in the greenhouse without getting any germination. The experiments described in the present paper deal mainly with the after-ripening and germination of seeds of *Cotoneaster divaricata* Rehd. & Wils. and *Cotoneaster horizontalis* Decne. A few preliminary tests with seeds of other species are also given.

Rosaceous seeds generally show marked delay in germination and require special treatment to after-ripen them and prepare them for germination (2, 3, 4, 5, 6, 9). The delay is due partly to dormancy within the embryo and partly to the effect of seed and fruit coats. Some rosaceous seeds germinate well after a period in the low temperature germinator for after-ripening followed by a higher temperature for germination as is reported by Crocker (2) for *Rosa*, by Giersbach and Crocker (9) for *Prunus americana*, and by Flemion (5) for *Sorbus aucuparia*. Flemion (6) found that seeds of *Rhodotypos kerrioides* germinate best if given one month at 25° or 30°C. in moist soil preceding low temperature treatment. For other rosaceous seeds mentioned above high temperatures preceding the low were not tried. Flemion's findings for *Rhodotypos* may hold for many rosaceous seeds. The favorable effect of high temperature period followed by low temperature has already been reported in the case of *Halesia carolina* (8) and *Tilia americana* (1). A period in a high temperature germinator previous to low temperature proved especially effective for seeds of *Symphoricarpos racemosus* (7). Pfeiffer (11) showed that a high temperature in the soil led to the decomposition of the tough coats by soil fungi, thus giving the embryos the proper condition for after-ripening at the low temperature. In other seeds, where the high temperature treatment preceding the low was beneficial, the effect of the high temperature seemed to be mainly on the coats for treatment with concentrated sulphuric acid displaced the need of the high temperature in the main. It has not been shown for most of the cases mentioned above that the high temperature is beneficial because of the decomposition of the coats by soil organisms. While this question is easy to answer for *Symphoricarpos*, where the coats are decom-

posed over the entire surface, it is difficult to settle for those rosaceous seeds in which the change occurs in a thin dehiscent layer which separates the pericarp into the two valves or for *Cotoneaster*, where it occurs in a thinner part of the coat on the upper side of the seed near the micropylar end which tears open before germination may begin.

EXPERIMENTAL RESULTS

PRELIMINARY TESTS

Seeds of various species of *Cotoneaster* were mixed with moist granulated peat moss and placed at 10°C. After-ripening and germination occurred as shown in Table I. It will be seen that seeds of *C. dielsiana* Pritz. and *C. zabelii* Schneid. germinated with 74 and 72 per cent respectively after three months at 10°C., while for *C. horizontalis*, *C. horizontalis* var. *perpuscilla* Schneid., *C. acutifolia* Turcz., *C. apiculata* Rehd. & Wils., and *C. lucida* Schlecht., the germination did not exceed 15 per cent after 10 months at 10°C.

TABLE I

GERMINATION OF SEEDS OF VARIOUS COTONEASTER SPECIES IN MOIST GRANULATED PEAT MOSS AT 10°C.

Species	No. of seeds used	Per cent germination after months								
		2	3	4	5	6	7	8	9	10
<i>C. acutifolia</i>	300	0	0	0	0	2	2	4	13	15
<i>C. apiculata</i>	240	0	3	6	8	8	9	10	10	10
<i>C. dielsiana</i>	450	41	74	97	97	97	97	97	97	97
<i>C. horizontalis</i>	300	0	0	0	0	0	0	2	2	2
<i>C. horizontalis</i> var. <i>perpuscilla</i>	300	0	0	0	1	2	6	9	9	10
<i>C. lucida</i>	200	0	0	0	2	2	2	2	4	5
<i>C. zabelii</i> Coll. I	250	0	72	77	78	78	78	80	80	80
<i>C. zabelii</i> Coll. II	200	53	59	84	84	84	85	88	88	88

Samples of these seeds were also planted directly in flats in a mixture of soil, sand, and granulated peat moss. Plantings were made in February and were kept in open, board-covered, or mulched frames in a greenhouse at 21°C., and in a cold room at 10°C. The poor seedling production after the first winter in cold frames followed by good germination after the second winter indicated that cold temperature alone was ineffective in bringing about germination. The flat plantings in the cold room at 10°C. were kept there until June to give them a longer cold period. This treatment had no particular advantage over the cold frame condition where low temperature ended with the coming spring (Table II). The flats kept in the greenhouse continuously gave no germination.

COTONEASTER DIVARICATA

Coats intact. The cleaned and dried seeds were mixed with moist granulated peat moss and placed in electrically-controlled ovens at constant

TABLE II

SEEDLING PRODUCTION OF VARIOUS SPECIES OF COTONEASTER KEPT IN FLATS OVER WINTER IN 10°C. ROOM, IN 21°C. GREENHOUSE, AND IN OPEN, BOARD-COVERED, AND MULCHED COLD FRAMES

Species	Per cent seedling production in flats after										Per cent good embryos
	First winter					Second winter					
	10° C. room*	Open C.F.	Board-covered C.F.	Mulched C.F.	G. H. 21° C.	10° C. room*	Open C.F.	Board-covered C.F.	Mulched C.F.	G. H. 21° C.	
<i>C. acutifolia</i>	4	0	0	0	0	79	9	93	21	0	100
<i>C. divaricata</i>	0	0	1	0	0	54	0	49	12	0	92
<i>C. horizontalis</i> Coll. I	0	0	0	0	0	7	2	44	9	0	92
<i>C. horizontalis</i> Coll. II	0	0	0	1	0	30	1	38	19	0	78
<i>C. horizontalis</i> var. <i>perpuscilla</i>	14	3	7	6	0	37	10	22	24	0	90
<i>C. lucida</i>	3	1	0	2	0	67	10	59	24	0	100

* Flats removed to mulched frame after the first winter.

temperatures of 1°, 5°, and 10°C. with and without preceding high temperature periods at 15°, 20°, and 25°C. Also tests at daily and weekly alternating temperatures (1° to 5°, 1° to 10°, 1° to 15°, 1° to 20°, and 1° to 25°C.) were tried. Table III gives the results obtained from 5°C., the optimum low temperature, when combined with a preceding high temperature treatment. A constant temperature of 5°C. alone was not sufficient to break the dormancy of seeds of *Cotoneaster divaricata*. Fifteen degrees C., 20°C., and 25°C. were equally advantageous when applied for two or three months preceding the low temperature treatment. A period of one month at these temperatures was insufficient for best results. One degree C., used as the low temperature, proved as good as 5°C. for after-ripening and germination. Ten degrees C., however, was ineffective. The data obtained at 1°C. and 10°C. are not reported. Weekly and daily alternating temperatures were not favorable. Weekly alternations of 5° to 10°C. and 5° to 15°C. were best, and yet only 6 per cent germination was obtained. Also samples of 75 seeds each were removed from the peat in the ovens at monthly intervals and planted in soil in flats. Seedling production in the greenhouse from these samples was very poor.

Since germination at low temperatures (1° and 5°C.) was good and samples from the same seed lot did not germinate in the greenhouse, presumably fully after-ripened seeds germinated immediately at the low temperatures and left only partially after-ripened or dormant seeds for the sample plantings. Individual seeds of *Cotoneaster* vary in their dormancy as is shown by their gradual germination over a long period (Table III).

TABLE III

AFTER-RIPENING AND GERMINATION OF *COTONEASTER DIVARICATA* AND *C. HORIZONTALIS* AT 5°C. WITH AND WITHOUT PRECEDING HIGH TEMPERATURE TREATMENT

Species	High temperature		Per cent germination after months at 5°C.						
	°C.	Months	2	3	4	5	6	7	8
<i>C. divaricata</i> (90 per cent good embryos)	15	1	0	0	1	2	4	6	7
		2	0	1	4	7	12	12	12
		3	0	3	3	18	19	22	22
	20	1	0	0	0	0	2	5	5
		2	0	0	3	6	14	16	17
		3	0	2	2	14	16	20	21
	25	1	0	0	0	1	3	6	7
		2	0	0	2	6	11	12	12
		3	0	0	5	18	21	22	22
	None		0	0	0	0	1	2	2
<i>C. horizontalis</i> (62.7 per cent good embryos)	15	1	0	6	12	12	14	14	14
		2	1	6	21	28	29	29	29
		3	0	5	30	42	42	42	42
	20	1	0	9	21	25	29	29	29
		2	0	4	18	20	25	26	26
		3	0	1	22	35	36	36	36
	25	1	0	4	11	15	17	22	22
		2	0	1	3	11	12	12	12
		3	0	1	13	25	26	26	26
	None		1	1	13	21	23	28	38

Seeds were also planted in flats in a soil mixture of one-third granulated peat moss, one-third soil, and one-third sand at various times during the year. Such plantings were made in November and February and kept in the greenhouse and under various cold frame conditions. The results are shown in Table IV. Except for the mulched bed no seedlings appeared after the first winter. However, good seedling production was obtained after the second winter. The board-covered frame gave the best results. In the mulched bed seedlings started to grow under the mulch when the outside temperature did not yet allow the removal of the protecting cover. These early seedlings either decayed under the mulch or were weak and apt to damp off later on. At the time when the mulch could be removed empty patches in the flats indicated the early growth and death of seedlings (Fig. 1 A). The mulched frame probably could give results equal to the board-covered frame if close watch were kept for the appearance of seedlings during the late winter. Flats could then be removed to a greenhouse.

The effect of three months of storage at room temperature is also shown

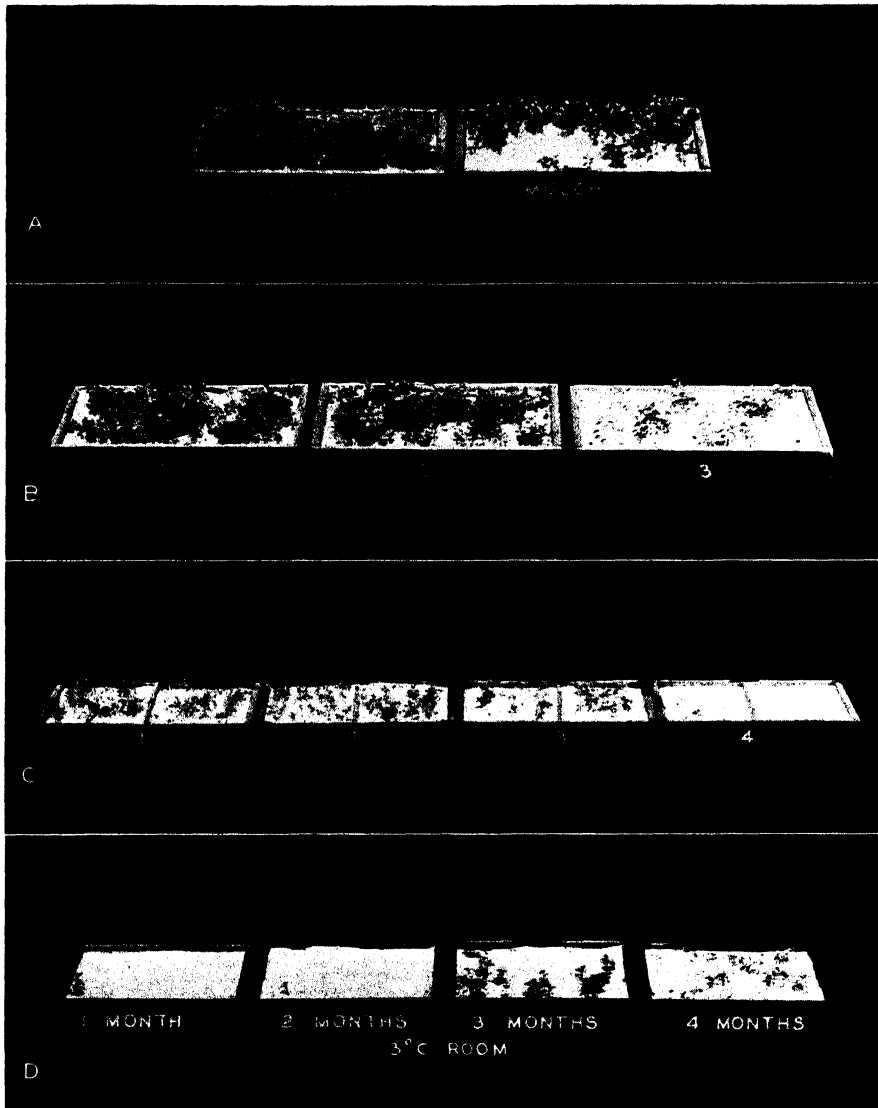


FIGURE 1. *C. divaricata*. (A) Seedling production in the cold frame after two winters. Seeds planted in November, 1930. (B) The effect of high temperature treatment followed by a winter in a board-covered cold frame. Preceding period in a greenhouse for (1) 6 months, (2) 5 months, (3) none. (C) Planted directly in cold frame (1) April 1, (2) June 1, (3) September 1, and (4) November 1. (D) Seedling production in the greenhouse after three months' pre-treatment in 13°C. greenhouse followed by 1, 2, 3, and 4 months at 3°C.

in Table IV. Seedling production for these stored seeds was slightly higher than for fresh seeds. This is due to partial after-ripening in dry storage (5, 6). Plantings in flats kept for various periods in greenhouses of different temperatures and then transferred to the board-covered frame for the winter gave good seedling production the following spring (Fig. 1 B).

TABLE IV

EFFECT OF A PERIOD OF DRY STORAGE OF SEEDS ON SEEDLING PRODUCTION OF *COTONEASTER DIVARICATA* IN FLATS IN COLD FRAMES AND GREENHOUSE; 96.5 PER CENT OF SEEDS WITH EMBRYOS; DUPLICATE LOTS OF 1500 SEEDS WERE USED (1929 CROP)

Treatment		Per cent seedling production of seeds planted			
		Immediately after harvest		After 3 months of storage at room temperature	
		Spring 1930	Spring 1931	Spring 1930	Spring 1931
Open	A	0	30	0	50
	B	0	41	0	24
Board-covered	A	0	49	0	75
	B	0	50	0	72
Mulched	A	11	32*	0	69
	B	2	24*	0	56*
21°C. Greenhouse	A	0	0	—	—
	B	0	0	—	—

* Seedlings showed injury from damping off when mulch was removed.

The flats shown were planted in August, 1931. Seeds planted in flat (Fig. 1 B 1) were kept in the greenhouse at 21°C. for six months and gave 29 per cent seedling production in the board-covered frame in the spring. These received approximately two months of cold. Flat (Fig. 1 B 2) with five months of greenhouse treatment gave 31 per cent and flat (Fig. 1 B 3) which had been constantly in the board-covered frame gave 19 per cent. It should be noted that the flat kept in the cold frame constantly received high temperature treatment from August to the beginning of winter. Figure 1 C and Table V show seedling production from successive tests made during the summer when duplicate flats each containing 400 seeds were planted monthly beginning with the first of March. April and May proved to be the optimum planting periods for *C. divaricata*. This confirmed the results of oven tests (Table III) which showed that high temperature followed by low brought about the after-ripening of *C. divaricata* seeds. When the seeds are planted in the spring so as to give several months of high temperature in the seed bed preceding the low temperature, only one winter is required for after-ripening and germination.

Table VI represents the percentage of seedling production from seeds planted directly in flats and kept for various periods in cold rooms with

TABLE V

SEEDLING PRODUCTION OF COTONEASTER DIVARICATA AND *C. HORIZONTALIS* FROM SUCCESSIVE MONTHLY PLANTINGS IN FLATS OUTSIDE AFTER ONE WINTER IN A BOARD-COVERED COLD FRAME; 90 PER CENT OF *C. DIVARICATA* AND 62.7 PER CENT OF *C. HORIZONTALIS* SEEDS HAD GOOD EMBRYOS; DUPLICATE FLATS OF 400 SEEDS WERE USED

Date of planting, 1932	<i>C. divaricata</i>	<i>C. horizontalis</i>	
		Crop A	Crop B
	Per cent seedling production, spring 1933		
September 1	44	28	—
April 1	61	37	—
May 1	52	30	29
June 1	32	31	31
July 1	40	27	23
August 1	28	29	28
September 1	19	33	19
October 1	19	31	19
November 1	1	—	4

TABLE VI

SEEDLING PRODUCTION OF COTONEASTER DIVARICATA SEEDS SOWN IN FLATS AND PLACED IN 3° AND 5°C. ROOMS AFTER VARIOUS PERIODS IN HIGHER TEMPERATURE ROOMS; 90 PER CENT GOOD EMBRYOS; DUPLICATE FLATS OF 400 SEEDS WERE USED

Initial warm room or greenhouse treatment		Per cent seedling production in flats after											
		Months at 3°C.						Months at 5°C.					
Temp. °C.	Months	1	2	3	4	5	6	1	2	3	4	5	6
15° room	1	1	0	1	1	7	9	0	0	5	9	26	35
	2	2	1	3	9	13	—	—	—	—	—	—	—
	3	0	0	3	15	—	—	0	1	13	12	—	—
	4	0	0	14	80	—	—	—	—	—	—	—	—
13° greenhouse	1	1	1	1	4	2	7	0	0	4	11	26	27
	2	1	3	12	20	26	—	—	—	—	—	—	—
	3	1	1	16	34	—	—	0	1	35	36	—	—
	4	0	1	19	70	—	—	—	—	—	—	—	—
21° greenhouse	1	0	1	2	5	9	13	0	0	2	12	18	22
	2	2	2	14	36	26	—	—	—	—	—	—	—
	3	0	1	9	27	—	—	0	0	2	75	—	—
	4	0	0	1	53	—	—	—	—	—	—	—	—
No high temperature		—	—	1	0	0	0	—	—	1	1	1	2

and without preceding high temperature treatment. The 13°C. greenhouse and the 15°C. room treatments seemed to be slightly more effective as a pre-treatment to low temperature after-ripening than the 21°C. greenhouse. Figure 1 D shows the effect of three months in the greenhouse at 13°C. followed by one to four months at 3°C. Seedlings developed after flats were removed from the cold room to a warm temperature. Four

months or more at a high temperature preceding a four-month period at low temperature is needed to obtain 70 to 80 per cent seedling production.

Coats opened over hypocotyl. Davis and Rose (4) reported for *Crataegus mollis* that seeds with the testa removed germinated up to 50 to 80 per cent after 96 days at 5° to 6°C., while no germination occurred with coats intact after 242 days. Freshly-harvested seeds of *C. divaricata* were opened

TABLE VII

EFFECT OF OPENING SEED COATS ON THE AFTER-RIPENING AND GERMINATION OF *C. DIVARICATA* AND *C. HORIZONTALIS* IN MOIST GRANULATED PEAT MOSS AT VARIOUS TEMPERATURES

Temp. °C.		<i>C. divaricata</i>						<i>C. horizontalis</i>					
		Per cent germination after months											
		3	4	5	6	7	8	3	4	5	6	7	8
1	Open Intact	0 0	10 0	50 0	51 0	53 0	53 0	0 0	10 0	53 1	67 4	68 4	68 7
5	Open Intact	0 0	27 0	58 1	59 1	58 1	59 1	0 0	27 3	65 10	67 14	67 17	67 19
10	Open Intact	0 0	0 0	0 0	2 0	37 2	63 5	7 2	15 5	19 7	30 13	55 22	62 25
15	Open Intact	1 0	1 0	1 0	1 0	1 0	1 0	0 0	0 0	7 0	8 0	10* 0	10 0
20	Open Intact	1 0	1 0	2 0	2 0	2 0	2 0	0 0	1 0	2** 0	2 0	2 0	2 0
25	Open Intact	3** 0	3 0	4 0	4 0	4 0	4 0	0 0	0 0	0 0	0 0	0 0	0 0

* Three per cent abnormal germination; ** one per cent abnormal germination (i.e., no hypocotyl growth).

by removing a part of the coat over the radicle with a sharp scalpel, since this part is thinner than the other parts of the coat. These seeds, when mixed with moist granulated peat moss and kept for five months at low temperatures (1°, 5°, or 10°C.), gave 50 to 58 per cent germination (Table VII). Higher temperatures (15°, 20°, or 25°C.) were ineffective. The same results were obtained after three or four months for one-year-old seeds. The germinated seeds were planted in pots in the greenhouse. Only one seed germinated at 15°C. and was too weak to be planted in soil. The few seedlings from 20°C. and 25°C. were weak and died. The numerous seedlings from 1°, 5°, and 10°C., however, grew into plants which appeared normal, although some of the cotyledons pushed through the soil with their seed coats attached. Some of these coats dropped off after a month or more while others remained attached and the shoots grew out at the side. Seeds which had been stored at room temperature for five months

were opened and given low temperature treatment. Sample plantings from these seeds gave 33 per cent seedling production after three months at 5°C. and 4 per cent after the same period at 1°C. No germination occurred from intact seeds. Higher germination of seeds with opened coats would doubtless have occurred had it not been for unavoidable injury to some of the embryos when the coats were opened. The slightest injury always resulted in decay.

The fact that opening the seed coats favors germination to such a degree proves that the dormancy of *C. divaricata* is at least partially due to a heavy coat.

Coats treated with concentrated sulphuric acid. Seeds of *C. divaricata* were treated with concentrated sulphuric acid for various periods, thoroughly washed, and placed in peat at 5°C. Table VIII shows that the effect of the acid treatment on the germination started with a one-hour period and increased favorably up to 2.5 hours, when 55 per cent germination was obtained after 100 days at 5°C. At that time the controls had not yet

TABLE VIII

GERMINATION OF SEEDS OF COTONEASTER DIVARICATA AND *C. HORIZONTALIS* IN PEAT AT 5°C. AFTER VARIOUS TREATMENTS WITH CONCENTRATED SULPHURIC ACID; DUPLICATE SAMPLES OF 300 SEEDS WERE USED

Species	Length of treatment in conc. H ₂ SO ₄	Per cent germination after days at 5°C.			
		80	87	94	100
<i>C. divaricata</i>	15 min.	0	0	0	0
	30 min.	0	0	0	1
	1 hour	0	2	4	6
	1.5 hours	1	7	17	21
	2 hours	2	14	24	29
	2.5 hours	1	23	50	55
	No treatment	0	0	0	0
<i>C. horizontalis</i>	15 min.	7	17	27	31
	30 min.	9	19	36	39
	45 min.	19	32	50	55
	1 hour	14	36	53	61
	1.5 hours	15	42	69	76
	No treatment	2	4	7	8

started to germinate. The germinated seeds were planted in soil in the greenhouse and grew into normal seedlings. Preliminary cutting tests of seeds treated with concentrated H₂SO₄ had shown that a period of more than 2.5 hours destroyed the outer coats and apparently injured the inner coats. However, as seeds with a 2.5-hour treatment still showed an increase in germination, tests with longer periods were started. These experiments are not yet completed. The data given in Table VIII indicate clearly that sulphuric acid treatment of the seed coats replaces the need for high tem-

TABLE IX

SEEDLING PRODUCTION OF *COTONEASTER HORIZONTALIS* FROM SEEDS PRE-TREATED AT HIGH TEMPERATURES FOLLOWED BY LOW TEMPERATURES OR AT CONSTANT LOW TEMPERATURES PRIOR TO PLANTING IN THE GREENHOUSE; 62.7 PER CENT OF SEEDS HAD GOOD EMBRYOS

Pre-treatment			Per cent seedling production in the greenhouse after months at low temperature				
High temp.		Low temp. °C.					
°C.	Months			1	2	3	4
10	1	1	2	6	16	10	10
	2	1	10	2	38	10	8
	3	1	6	16	18	10	0
15	1	1	2	12	12	8	6
		5	0	18	16	8	8
		10	0	0	0	0	0
	2	1	2	32	36	28	4
		5	2	24	24	4	2
		10	0	0	0	0	0
3	1	0	16	42	42	2	
	5	4	14	20	20	0	
	10	0	2	2	2	0	
20	1	1	0	14	20	20	14
		5	0	10	8	10	2
		10	0	0	0	0	0
	2	1	6	24	50	24	12
		5	4	42	32	10	4
		10	0	0	0	0	0
3	1	2	22	42	36	8	
	5	2	16	10	12	0	
	10	0	0	0	0	0	
25	1	1	0	4	24	16	8
		5	0	2	22	6	12
		10	0	0	0	0	0
	2	1	4	12	30	32	16
		5	2	32	30	12	0
		10	0	0	0	0	0
3	1	0	32	22	24	16	
	5	0	24	24	12	6	
	10	0	0	0	0	0	
No high temperature		1	0	0	8	8	4
		5	0	2	6	4	0
		10	2	0	0	0	0

perature treatment. Both of these treatments serve to overcome the inhibiting effect of the seed coat on after-ripening.

Excised embryos. Treatments of coats with concentrated sulphuric acid may also be used as part of a method to remove the outer and inner seed coats entirely. When seeds of *C. divaricata* treated with concentrated

H₂SO₄ for 2.5 hours were washed thoroughly and soaked in water overnight, the outer coat and the inner coat with the adhering endosperm could be removed.

The excised embryos were placed on moist filter paper and in water in bottles through which air was passed. Out of 25 embryos placed on moist filter paper only six developed the epicotyl. No hypocotyl growth occurred. After two weeks the embryos in aerated water showed slight growth of the epicotyls and also elongation of the hypocotyls. There was no root hair development. These embryos were planted in pots in the greenhouse, where growth was very slow and stunted. Most of the seedlings were dead after 10 days in the greenhouse.

COTONEASTER HORIZONTALIS

Coats intact. The experiments on *C. horizontalis* were conducted in the same way as the ones on *C. divaricata*. Preliminary tests (Table I) had shown that low temperature alone was not sufficient to bring about complete germination of *C. horizontalis* seeds. However, the inefficiency of the low temperature varied with different crops. Table III shows the germination at the optimum low temperature (5°C.) with and without preceding high temperature treatment. In the case of the crop of *C. horizontalis* reported in this table, there is little difference between the effectiveness of constant low temperature and high followed by low temperature treatment whereas the initial high temperature treatment was very beneficial for *C. divaricata*. The seed coats of this species were less hard. One degree C. was not quite so effective as 5°C., while 10°C. was ineffective. For the high temperature treatment, 15° and 20°C. seemed to be slightly better than 25°C., and two or three months at these temperatures better than one month.

Weekly and daily alternating temperatures did not show better results than constant low temperature. Weekly alternations of 1° to 5°C., 1° to 10°C., and 1° to 15°C. resulted in 9, 20, and 16 per cent germination after six months and were slightly more effective than the same temperature combinations as daily alternations after the same period (6 per cent for 1° to 5°C., 16 per cent for 1° to 10°C., and 8 per cent for 1° to 15°C.)

Sample plantings in the greenhouse from low temperatures with and without preceding high temperature gave good results (Table IX and Fig. 2 B). Since only 62.7 per cent of the seeds of this crop had good embryos the germination percentages are considerably higher than they appear.

Again seeds were planted in a soil mixture of one-third soil, one-third sand, and one-third granulated peat moss. When seeds were planted in flats in the fall and kept outside under various cold frame conditions no seedling production occurred after the first winter. However, up to 36 per cent seedling production was obtained after the second winter. The

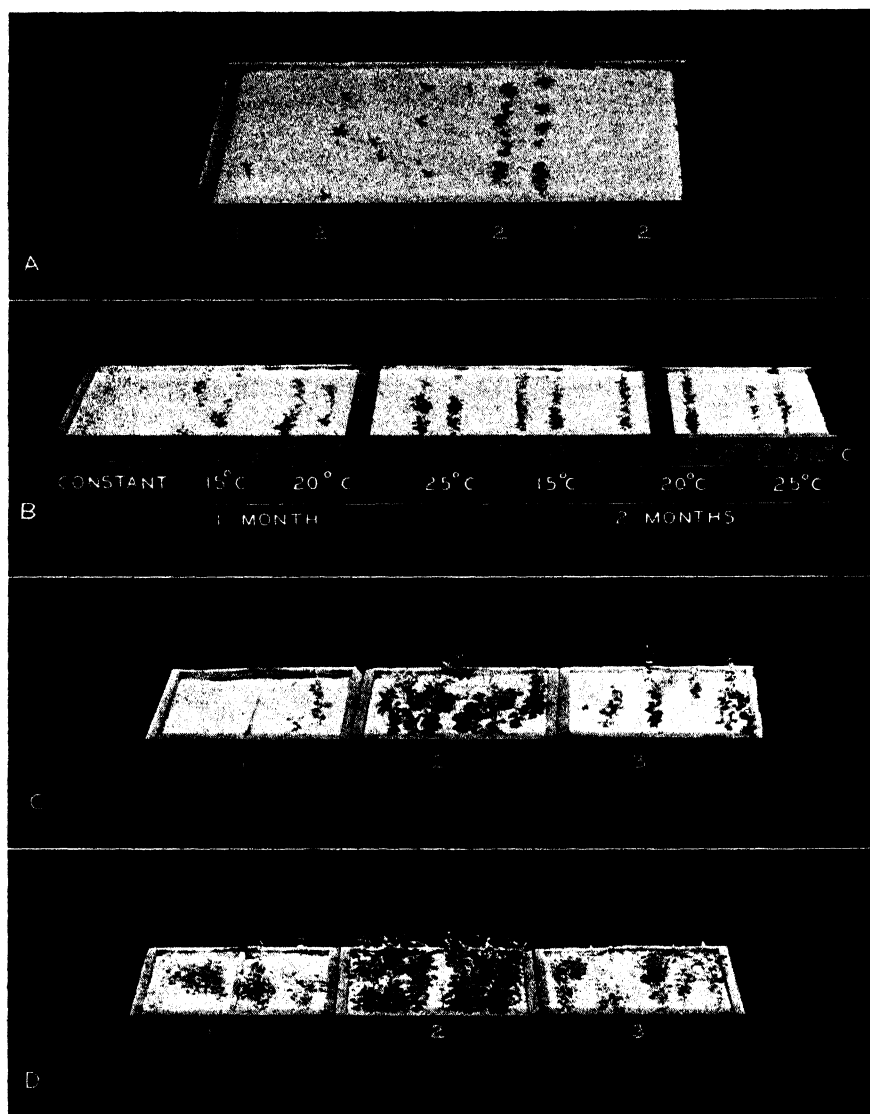


FIGURE 2. *C. horizontalis*. (A) Seedling production in the greenhouse from sample plantings after three months at low temperatures: (1) coats intact, (2) coats opened (from left to right: 1°C., 5°C., and control). (B) Seedling production from sample plantings in the greenhouse after four months' pre-treatment: (1 or 2 months at 15°, 20°, or 25°C. followed by three or two months at 1°, 5°, or 10°C. or four months at constant 1°, 5°, or 10°C.). (C) Seedling production in cold frames after one winter: (1) open, (2) board-covered, (3) mulched. (D) Mulched frame with one month pre-treatment at higher temperatures: (1) 15°C. room, (2) 13°C. greenhouse, and (3) 21°C. greenhouse.

TABLE X

SEEDLING PRODUCTION OF *C. HORIZONTALIS* OUTSIDE AFTER VARIOUS PERIODS IN DIFFERENT COLD ROOMS WITH AND WITHOUT PRECEDING HIGH TEMPERATURE TREATMENT; 62.7 PER CENT OF SEEDS HAD GOOD EMBRYOS; DUPLICATE SAMPLES OF 200 SEEDS WERE USED

High temperature treatment		Per cent germination in flats											
Temp. °C.	Months	Months 3°C.						Months 5°C.					
		1	2	3	4	5	6	1	2	3	4	5	6
15° room	1	5	6	4	6	4	7	3	4	4	5	14	10
	2	4	2	3	—	—	—	—	—	—	—	—	—
	3	0	0	4	7	—	—	3	4	3	7	—	—
	4	0	0	2	—	—	—	0	0	3	5	—	—
13° greenhouse	1	11	5	8	11	7	9	4	4	7	13	27	15
	2	6	6	19	—	—	—	—	—	—	—	—	—
	3	1	6	17	12	—	—	1	2	9	10	—	—
	4	0	4	26	—	—	—	0	0	4	44	—	—
21° greenhouse	1	5	4	9	5	7	0	1	1	8	5	19	8
	2	0	2	7	—	—	—	—	—	—	—	—	—
	3	0	0	12	2	—	—	0	0	2	1	—	—
	4	0	2	12	—	—	—	0	0	1	11	—	—
No high temperature		—	—	3	6	8	—	—	—	4	8	10	—

results were similar to those for *C. divaricata*. The best results were obtained with the board-covered frame. These results led to the planting of duplicates of 400 seeds each, at monthly intervals beginning on May 1st. Table V shows that May is the best month for outside plantings of *C. horizontalis*. A comparison of the two crops of *C. horizontalis* (Table V) indicates that the degree of dormancy varies for different crops. Other tests not reported led to a similar conclusion. When seeds in flats were pre-treated in greenhouses of 13° or 21°C. or in a warm room of 15°C. for one month, seedling production in the board-covered frame was considerably increased. The open, board-covered, and mulched frames gave 2, 11, and 5 per cent germination (Fig. 2 C), while with one preceding month in the 15°C. room or in greenhouses of 13° and 21°C. seedling production in the mulched frame went up to 15, 26, and 10 per cent, respectively (Fig. 2 D and Table X).

Although the seeds of this crop gave a rather high germination without a preceding high temperature treatment, it will be noticed that the best seedling production resulted from a pre-treatment of four months at 13°C. followed by three months at 3°C. or four months at 5°C. The inconsistencies in the results in this case were due to the fact that the seed coats were more permeable than in the other crops studied.

Coats opened over hypocotyl. When seed coats of *C. horizontalis* were removed over hypocotyl, three or four months of low temperature were required to after-ripen the seeds (Table VII). Without the inhibiting effect

of the seed coat the embryos of *C. horizontalis* and *C. divaricata* showed the same dormancy. Figure 2 A shows seedling production of sample plantings of opened and intact seeds of *C. horizontalis* in the greenhouse at 21°C. After three months at 1°C. 11 per cent seedling production for opened and 1 per cent for intact seeds were obtained. After three months at 5°C. 43 per cent for opened and 5 per cent for intact seeds were obtained.

Coats treated with concentrated sulphuric acid. Seeds of *C. horizontalis* were treated with concentrated sulphuric acid for various periods, washed, and placed in moist peat moss at 5°C. Table VIII shows that the effect of the acid treatment of seed coats on germination began with a period of 15 minutes and increased favorably up to 1.5 hours, when 76 per cent germination was obtained after 100 days in moist peat moss at 5°C. Preliminary cutting tests of seeds treated for various periods had shown that seeds treated longer than 1.5 hours appeared to be injured. However, as seeds with a 1.5-hour treatment still showed an increase in germination tests with longer periods were begun. The destruction of the seed coat with H₂SO₄ substituted for the high temperature period otherwise needed for obtaining good seedling production.

Excised embryos. Again the sulphuric acid treatment made possible the removal of the entire coats. Seeds were treated with concentrated H₂SO₄ for 1.5 hours, washed, and soaked overnight. Then the outer coat and the inner coat with the adhering endosperm were removed.

The excised embryos were placed on moist filter paper and in water in glass bottles through which air was passed. In the case of the petri dish treatment, neither epicotyls nor hypocotyls developed. Cotyledons became enlarged, the one close to the filter paper more than the one away from it.

In aerated water all embryos showed slight development of the epicotyl as well as the hypocotyl. The root, however, did not show root hair development. When planted in the greenhouse after two weeks, only very slow growth of the epicotyl occurred, compared with seedlings from seeds after-ripened at low temperature. Most of the seedlings were dead after 10 days in the greenhouse.

DISCUSSION AND SUMMARY

1. Seeds of *C. dielsiana* and *C. zabelii* give nearly 100 per cent germination after four months in moist granulated peat at 10°C. Seeds of *C. acutifolia*, *C. apiculata*, *C. horizontalis*, and *C. lucida* give very poor germination even after 10 months in the same condition.

2. If seeds of the four last named species are planted in flats in the fall and placed in board-covered cold frames they give very few seedlings the following spring. If the seeds are kept in the cold frames for two winters and the intervening summer they give excellent seedling production the

second spring. The dormancy of these seeds is due to two factors: (a) the coat factor which is overcome by a period of 3 or 4 months in soil at 15° to 25°C., and (b) after-ripening of the dormant embryos which is brought about by a period of 3 or 4 months in a germinator at 1° to 10°C. The coat factor must be disposed of first in order to insure prompt after-ripening of the embryos. The optimum temperature for after-ripening of the embryos is 1° to 5°C. Ten degrees is much less effective while 15°C. is without effect.

3. Seeds planted in flats in the spring and placed in cold frames give good seedling production the following spring, especially if the flats are protected by board covers during the winter. This gives an initial warm period in the soil which overcomes the coat effect and a later cold period which after-ripens the embryos.

4. The coat effect can also be overcome by removing the coats over the hypocotyl or by treating the seeds of *C. divaricata* for 2.5 hours and *C. horizontalis* for 1.5 hours with concentrated sulphuric acid. These coat treatments entirely displace the need of the initial high temperature period in the soil.

5. An initial high temperature period in the soil to overcome coat effects followed by a low temperature period for after-ripening the dormant embryos has been found beneficial by other authors for the germination of *Rhodotypos kerrioides* (6), *Ilalesia carolina* (8), *Tilia americana* (1), and *Symphoricarpos racemosus* (7). In *Symphoricarpos racemosus* seeds it was shown that during the high temperature period soil fungi decompose the tough seed coats and thereby remove the coat effects (11). For the other cases, including *Cotoneaster* seeds, it is not known whether soil organisms play a part or whether physical and chemical changes in the coats quite independent of the action of soil organisms prove effective.

6. A good practical method for seedling production of *C. divaricata* and *C. horizontalis* is planting of seed in flats in April or May and keeping these in a board-covered frame over winter. Good seedling production will occur the following spring. However, seeds may also be planted immediately after harvest. In that case, a good crop of seedlings will be produced in the spring after the second winter. Both methods may be used by nurserymen. Treatment of seeds with concentrated H₂SO₄, which will substitute for the high temperature period, combined with low temperature treatment shows possibilities for practical use. However, further studies are needed to develop the details of this method.

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INITIATION OF THE ASCOCARP AND ASSOCIATED PHENOMENA IN COCCOMYCES HIEMALIS¹

MYRON P. BACKUS²

INTRODUCTION

A general account of the development of the cherry leaf-spot pathogen, *Coccomyces hiemalis* Higgins, was published by Higgins (45) in 1914, shortly after he had discovered the ascigerous stage of the organism and described the fungus as a new species.

Within the last two years research has brought to light significant new facts concerning sexual reproduction in certain of the Ascomycetes. The recent and rather spectacular demonstrations by Drayton (36, 37), Dodge (35), and Ames (7), that the microconidia (spermatia) of certain species with which they have worked serve in a fertilizing capacity, represent an outstanding contribution to mycological knowledge, and they have afforded a parallel to the well-known work of Craigie (28, 29) and others (2, 3, 4, 5, 6, 8) on the function of the spermatia in the rusts. Spermatia (pycniospores, microconidia) characterize prominently the great groups of the red algae, the rusts, and the Ascomycetes (including those involved in lichen associations).

The term "spermatium" (from σπερμάτιον) was introduced by Tulasne (66) in connection with his early studies on the lichen fungi. It was intended that the word should carry the implication of maleness, which property Tulasne suspected the bodies to which he applied it to possess. It has been conspicuous in all the ascomycete and rust literature since his time. Arthur (9), however, proposed "pycniospore" to replace "spermatium" in the case of the rusts, and his terminology has had considerable following. In the Ascomycetes the term "microconidium" has come into quite common

¹ The work here presented was begun in the Graduate School of the University of Wisconsin, Madison, Wisconsin, but the major portion of it was done at the New York Botanical Garden, New York City, during tenure of a National Research Fellowship in the Biological Sciences, and at the Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, where the writer began carrying on mycological studies in September, 1933.

² The author is greatly indebted to Prof. E. M. Gilbert of the University of Wisconsin for suggesting this problem and for encouragement and many helpful suggestions in the early stages of the investigation; to Dr. B. O. Dodge of the New York Botanical Garden who sponsored the work executed during tenure of a National Research Fellowship and who gave much useful advice both during that period and also during the recent months when the study was brought to completion; to Prof. R. A. Harper of Columbia University for valuable counsel on various points; to Prof. G. W. Keitt of the University of Wisconsin for his stimulating interest in the problem and for providing certain materials which aided progress on the work; finally to Dr. B. O. Dodge and Prof. R. A. Harper for critical reading of the manuscript.

usage. Originally coined by Tulasne and Tulasne (67) to designate any spermatiform spore which would germinate, as distinct from "spermatia" which would not germinate, it was later used indiscriminately by the disciples of Brefeld to label any minute spermatiform bodies, on the grounds of their belief that all so-called "spermatia" were really only reduced conidia. In more recent ascomycete literature, except in the case of that dealing with lichens and Laboulbeniales where "spermatium" has almost completely predominated, "microconidium" and "spermatium" have been used more or less interchangeably without the authors in either case necessarily intending to commit themselves thereby as to their ideas concerning the real nature of the bodies in question, although naturally many of those who have favored the view that they represent entirely asexual structures have avoided the word spermatium. There seems every reason to believe, however, that the pycniospores of the rusts and most of the "microconidia" of the Ascomycetes are structures entirely homologous with one another and homologous also with the red algal spermatia.

In view of the proof recently brought forth that the microconidia of certain ascomycetous forms actually function to effect a fertilization, all so-called microconidium-producing forms among the Ascomycetes now naturally claim special attention. Higgins' work on *Coccomyces hiemalis* established this as an interesting microconidium-producing species, but because of its general nature did not include anything like a thorough-going investigation of the possible relation of the spermatial bodies to the initiation of the ascocarpic stage of the fungus.

The archicarps of *Coccomyces* show a marked resemblance to those of some of the lichens, not only as regards their form and method of development but also in the positions assumed by the trichogynes relative to the spermatial fruiting bodies. Students of the fungi know how prominently the morphology of the sexual mechanism in the lichens has figured in some of the leading theories as to the phylogeny of the group as also in the development of our ideas concerning the nature of spermatial bodies in other Ascomycetes.

From the morphological study which is here presented, it seems clear that there are definite trichogyne-microconidium relations in *Coccomyces*, though the facts of nuclear and cell fusions still need further investigation. The findings here reported furnish a basis for further cytological and experimental studies, already under way, and which it is hoped may contribute to settling the question of the functional rôle of the spermatia in the Ascomycetes and its relation to that in rusts and algae.

LITERATURE REVIEW

Although conclusive proof that spermatial bodies in the Ascomycetes may function in reproduction is recent, yet as early as the middle of the

last century a vague notion was put forth by Itzigsohn (48, 49) that in the case of the lichens the small bodies in question are male elements. Itzigsohn first drew the attention of botanists to those conceptacles on the lichen thalli which we now know as spermogonia, and held that they corresponded to the antherids of mosses and hepatics. He had, however, very erroneous ideas concerning the appearance and method of origin of the small corpuscles seen inside of the spermogonia, believing that they were motile like the spermatozoids of the Bryophytes and that they were formed as these cells are.

Tulasne (66) a little later correctly described the bodies produced in the Itzigsohnian conceptacles and gave an accurate account of their formation. He applied the name "spermatics" to the small elements produced in the conceptacles and the name "spermogonie" to the conceptacle itself. He was impressed by the small size of the spermatia and by their refusal to germinate, and he felt that some day "it would be demonstrated that there resided in them a certain force or nature like that of pollen" (67, p. 183). Moreover, with his understanding of the relation of the Ascolichens to the autonomous Ascomycetes, he was quick to carry his terminology and ideas to the latter group. Soon he met with difficulties, however, for he found that some of the small bodies which he was calling "spermatics" germinated with the greatest ease and in every way acted like ordinary spores. He was undoubtedly confused and somewhat bewildered by this; but it seems certain that even in these to him perplexing circumstances he did not by any means entirely give up the idea that at least those small bodies which, as the lichen spermatia, consistently refused to germinate (according to his later definition only such could be called "spermatics") would eventually be demonstrated to act as male elements.

Stahl (62, 63) went further. Not only did he believe that the spermatia of the lichen fungi accomplish a fertilization, but he described the process. He figured the attachment of spermatial bodies to the tips of trichogynes which projected above the surface of the thallus and which could be followed down into the thallus where they could be seen to arise each as a prolongation of a coiled archicarp. He observed an opening between the fused spermatium and trichogyne-end, and gelatinization of the trichogyne cross-walls which he reported as occurring subsequent to the attachment of spermatia. His findings were substantiated later by Baur (14) and others who observed similar fusions of spermatia with trichogynes.

A cytological study by Bachmann (10, 11) of a peculiar form of *Collema pulposum* revealed a most interesting situation in the relations of spermatia to fertilization. The archicarps occur in groups. Each is made up of a coiled basal portion of fifteen or more cells and a long multiseptate trichogyne. The spermatia in this form are not produced in large and highly organized spermogonia as is the case in most lichens. They are borne

loosely attached in small groups of from two to fourteen on a hypha which may be as deep as three hundred microns below the surface of the thallus. The trichogynes grow directly toward these cells, and their tips become appressed to and often coiled about them. Fusion between the two structures occurs, and it is concluded on the basis of cytological observations that nuclei derived from the spermatia probably pass down the trichogyne into the ascogonial region. The situation here shows important points of resemblance to that described by Dodge (31) in the case of *Ascobolus carbonarius* where a long tapering multicellular trichogyne is formed whose tip in some cases coils about a conidium-like structure—which phenomenon Dodge takes as an indication that at least some of the conidia are functionally equivalent to the spermatia of the lichens.

The number of morphological or cytological studies on lichens has not been large. The most extensive program of research of this kind ever undertaken was by Moreau and Moreau and certain others of the Dangeard school (53, 54, 55, 56, 57, 58, 59). It is rather curious that in their numerous studies, extending over a long period of years and covering a large number of forms, they were unable to find any indications that the spermatia play any part in the initiation of the ascocarpic stage. In fact they have concluded that in most cases the trichogynes do not even reach the exterior, hence such copulations as Stahl and Baur described would be impossible.

Among the autonomous Ascomycetes many species have long been known to show, like many of the lichens, prominent trichogynes as a part of the archicarpic apparatus, and also to produce spermatium-like bodies, often referred to as "microconidia." In the Laboulbeniales it has been taken for granted by most mycologists, on the basis of the work of Thaxter (64) in particular, that in most species development of the ascigerous stage depends upon the attachment of spermatia to the trichogynes and subsequent passage of nuclei down through this organ into the ascogonial region, although to date the only cytological studies in connection with the Laboulbeniales have been on species which are said not to produce spermatia (39).

In other groups of the Ascomycetes various investigators have studied spermatium-producing forms which closely resemble the lichens of the type studied by Stahl in the possession of long multiseptate trichogynes. In many of these the trichogynes are found to grow up in such a way that copulation with spermatia would be possible, but in most instances no such fusion was reported. In *Gnomonia erythrostoma* Brooks (23) did report finding spermatia attached to certain hyphae to which he referred as "trichogynes," although he found no evidence of nuclei passing into them. He points out that the trichogyne, though originally it may have served as a receptive organ for male nuclei, now may serve a different

purpose, perhaps acting as Van Tieghem (65) suggested, in a respiratory capacity. According to Likhité (51) Brooks was not dealing with real trichogynes.

Blackman and Welsford (17) investigated the development of *Poly stigma rubrum* and concluded that in this form both the trichogynes and spermatia are abortive. Higgins (46) studied two species of *Mycosphaerella*, *M. bolleana* and *M. personata*. In both species trichogynes with tapering tips prolong the coils and grow to the surface soon after formation of the spermogonia, but Higgins was unable to find any evidence that the spermatia play any part in the development of the perithecia. In another species, *Mycosphaerella cerasella* Aderh., recently studied by Jenkins (50), no indication was found of nuclei coming into the archicarpic system from an outside source. In *Diplocarpon rosae* and *D. earliana* Wolf (68) found trichogynes projecting above the leaf surface at the time microconidia were being formed, but failed to establish any further relations. In various species of *Coccomyces* Higgins (45) reported microconidia developing in what apparently were old macroconidial acervuli on infected regions of cherry and plum leaves. Production of these minute bodies was found only in the late summer and fall; and almost simultaneously with their appearance, according to his observations, stromata develop in the leaf tissue immediately below the acervuli. In these stromata coiled archicarps appear which are prolonged by trichogynes, the ends of which project above the leaf surface into the region where microconidia are produced. Higgins points out that if the microconidia function as sexual organs or have ever so functioned, their formation on the same stroma and at the same time as the coils would make fertilization more certain. But he was unable to find any indications that the microconidia ever come into intimate relation with the trichogyne ends.

It will be readily seen from the cases just cited that in the many years since Stahl's work little convincing evidence has been adduced, through morphological or cytological study, that would give one reason to infer that spermatial fertilization such as he concluded occurs in the lichen fungi he investigated, is of common occurrence, if it exists at all, in either the Ascolichens or any of the autonomous Ascomycetes.

It is of exceeding interest, then, that recently Drayton (36, 37), Dodge (35), and Ames (7), employing experimental methods involving single spore cultures, demonstrated conclusively that in *Sclerotinia gladioli*, *Neurospora sitophila*, *N. tetrasperma*, and *Pleurage anserina* the microconidia may act as fertilizing agents, thus vindicating the prediction made by Tulasne about seventy years before.

MATERIALS AND METHODS

This study was confined to *Coccomyces hiemalis* as it develops on a single host, *Prunus cerasus* L. Sour cherry leaves infected with the organism were

collected from the University of Wisconsin orchard at Madison, Wisconsin, during the summers and falls of 1929 and 1930. Private cherry orchards in that vicinity were also drawn upon. Collections were made from the University orchard of Cornell University at Ithaca, New York, in the fall of 1932; and in 1931 and 1932 additional material was obtained through the courtesy of J. G. Goodrich of Lockport, New York, from certain cherry orchards in Niagara and Wayne Counties, New York. These sources were supplemented by inoculating small potted cherry trees and utilizing infected leaves from these. In the fall of 1933 there was available a particularly satisfactory supply of such material. A large number of fixations of "spots" from infected leaves from all these sources were made, using a variety of fixatives, but Bouin's, Allen's Modification of Bouin's, and Formol-acetic alcohol were most extensively employed. From the imbedded leaf squares sections from 5 to 10 μ thick were cut and stained largely with iron-alum haematoxylin used in connection with various counterstains. A portion of the slides were stained with a safranin-fast green combination.

Slides prepared with microtome sections were supplemented by two other sorts of preparations: free-hand sections of fresh or hardened material stained with erythrosin, etc., and mounted in glycerine; and preparations made by staining hand sections of fresh material in aceto-carmin and teasing the material out with fine needles under a high-power dissecting microscope—a type of preparation previously used to advantage by the writer (12) in another connection.

OBSERVATIONS

PRELIMINARY CONSIDERATIONS

The ascocarps of *Coccomyces hiemalis* mature in the spring on overwintered cherry leaves on the ground; and the ascospores produced constitute the main source of inoculum for the initiation of the "leaf-spot" disease on the new foliage of the season. On each spot which develops, a typical *Cylindrosporium* acervulus, which is the asexual fruiting body of this organism, forms a few days after the fungus has effected entrance into the host. Most of these facts were adequately demonstrated by Higgins in 1914. Macroconidia are produced in large numbers in the acervuli under proper weather conditions and by means of these spores the fungus propagates itself from leaf to leaf and from tree to tree throughout the summer and into the fall as long as the leaves hang on the tree. It would appear that under normal conditions the foliage never becomes too old to be infected. Some of the heaviest infection which I ever observed took place in the middle of October. More will be said about these very late infections, for they are of particular interest in relation to the problems we are here concerned with.

From the time when the first infections develop in the spring, and on through most of the summer, a section through a mature "spot" will reveal the following essential features (see Fig. 1 A)³: intercellular mycelium spreading loosely through the leaf tissue from epidermis to epidermis and sending out haustoria into the cells of the mesophyll, the bundle sheaths, and occasionally into the epidermal cells; and an acervulus bearing long macroconidia which breaks through the lower epidermis. Sometimes a smaller acervulus may also be seen rupturing the upper epidermis. This we may call the typical "summer" picture in contrast to other appearances occasioned later by morphological changes of various kinds which will be described below.

Frequently the leaves bearing early infections drop early in the summer, especially if infection is heavy, and carry the fungus with them to an early death. The development of "shot-holes" under certain circumstances, the drying out of spots during hot and dry summer weather, etc., are other factors which tend to reduce the chances of early infections playing a direct part in the development of the perfect stage. It is the later infections with which we consequently are most concerned.

MICROCONIDIOPHORES AND MICROCONIDIA

The first deviation from the typical "summer" picture and the first sign of the approach of the initiation of the perfect stage is seen about the last of August, and is to be found in connection with the acervulus. In a few isolated cases here and there, particularly in spots on yellowing leaves, sections now reveal (Fig. 2 B to D; Fig. 5 A, E) that the macroconidiophores have been replaced by microconidiophores bearing microconidia in abundance. This change does not go on simultaneously throughout all spots by any means, nor does it seem to be closely correlated with any change in temperature, etc., incident to the approach of fall. It seems rather to depend on the appearance of certain physiological conditions in the leaf or in the individual infection. The yellowing leaves are likely to be the first in which microconidia develop, but microconidia are never seen in acervuli on diseased leaves which have turned yellow early in the summer; and in the fall, especially in October and later, green leaves as well as yellow ones may show microconidial production. As fall advances, the proportion of acervuli producing microconidia becomes increasingly greater. Examination of a group of samples taken at random in the middle of October is likely to show the majority of the acervuli producing microconidia. In a few there may be only macroconidia present; and some may

³ Attention is called to the fact that in this figure and in all of the rest of the illustrations in this paper where sections of leaves are pictured, the leaf is for psychological reasons shown in an inverted position, the lower surface being at the top.



FIGURE 1. (A) Cross-section through infected portion of a leaf, showing typical appearance of fungus in summer condition. Edge of acervulus visible at upper left. (B) Cross-section through a late infection. Microconidiophores beginning to form at extreme right side of the acervulus. Small stroma initials visible at *a* and *b*.

be just at the point of changing over to the production of microconidia. This change seems to occur fairly gradually in most cases, and for a short time both spore types may be formed simultaneously in the same acervulus.

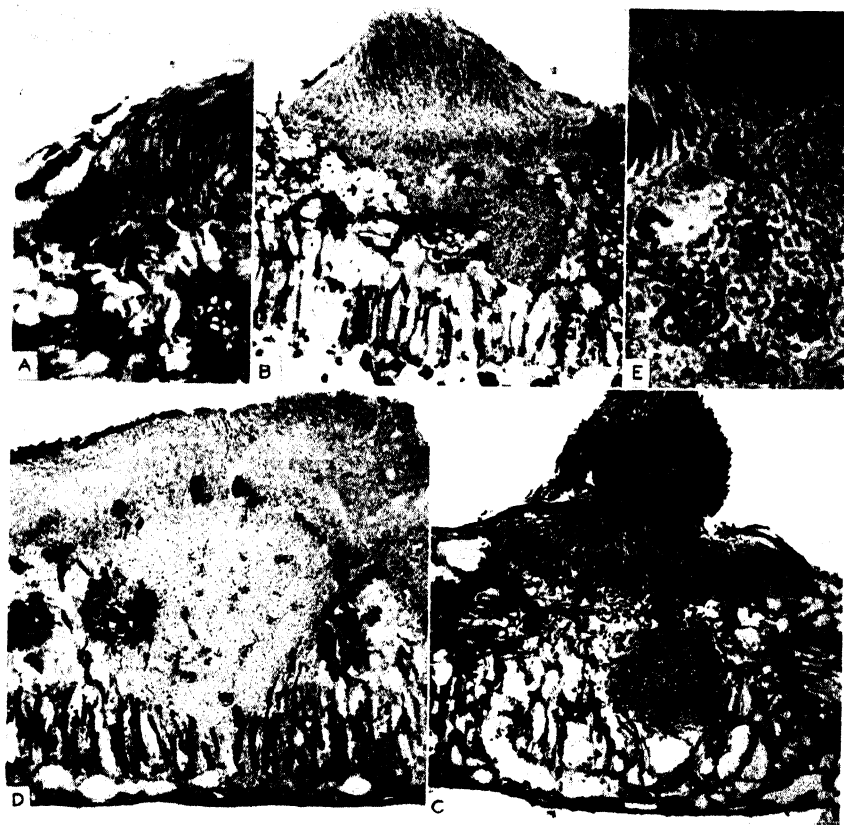


FIGURE 2. (A) Edge of a young acervulus from a late infection (artificial inoculation). Microconidiophores forming at extreme left margin. (B) Section through infected area of a leaf showing microconidia being produced in the acervulus, and old macroconidia overlying microconidial mass. A developing stroma is seen in the leaf tissue below the acervulus. \times about 140. (C) Acervulus changing over to microconidial production. (D) Section showing stroma extending through leaf almost to the upper epidermis. Coils visible in stroma. \times about 140. (E) Small portion of a section through a stroma in contact with acervulus base. Parts of four coils visible. Note trichogyne segments extending up toward microconidiophore layer.

Sometimes the change occurs first on one side of the fruiting body (Fig. 1 B); frequently it begins in the central region, which is the oldest part of that structure, since, as Higgins pointed out, the acervulus shows centrifugal development.

Certain inoculation experiments conducted in September on small experimental trees with unusually old foliage (the leaves were forced out in the greenhouse during the preceding winter), provided an interesting variation. Heavy infection was obtained, and the leaves started turning yellow within a week after the inoculation. It was found that occasionally an acervulus developed which began to produce macroconidia in regular fashion, for sections showed macroconidia produced in the central or oldest region; but apparently before the acervulus had completed its centrifugal development conditions favoring microconidial formation intervened, and microconidiophores developed directly in the peripheral regions without macroconidiophores having preceded them (Fig. 2 A). In such a case the microconidia appear first in the youngest portion of an acervulus. It is obvious that the situation is not a simple one and that wide variations occur, depending upon many interrelated factors—the physiological conditions in the substratum, the age of the infection, the age of the infected leaves, etc.

In view of these facts and the fact that in this organism the microconidia ordinarily form on the same stromatal base which earlier produced the asexual *Cylindrosporium* spores, it was thought worthwhile to attempt to discover whether any cases occur in which fruiting bodies of a purely spermatial or microconidial type, comparable perhaps to the spermogonia of the lichens, develop. That such cases are to be found was ascertained by observing that in certain young fruiting bodies where the epidermis had not yet been broken, only microconidiophores were present; here the possibility of macroconidia having been produced earlier was excluded. These cases are rare, however, and would seem to be chiefly confined to very late infections. Moreover, such fructifications are always of small diameter. It should be stated that there is a strong tendency for infections even very late in the season to develop first a typical macroconidial acervulus. Some trees which were kept under observation during the past fall (1933) showed extremely heavy secondary infection during the latter part of October, following a late inoculation. Examination revealed that most of the young spots even at this late date developed acervuli bearing "summer" conidia. But the production of these spores is, of course, of very limited duration under such circumstances. In this particular instance, however, some of the acervuli never did produce microconidia because in some cases before the acervuli even reached maturity, low temperatures killed the infected leaves.

The microconidiophores are branched structures varying considerably both in size and form. They range from very simple types to rather complex branching systems as is illustrated in Figure 3 A to R. They average about 25μ in height. It was found possible to study these structures to advantage by making free-hand sections through acervuli bearing them and

teasing out such material in aceto-carmin. The microconidiophores show in such preparations a rather definite type of branching—which is typical also of the vegetative mycelium, to a certain extent, of the paraphyses, and in fact wherever branching in this organism was observed. Typically a branch grows out just below a septum, and a cross-wall frequently appears in the branch near the point of origin. As a variation of this a three-way forking may occur. The branches of the microconidiophores are not by any means all of the same diameter. Some of them are extremely thin, as can readily be seen from an examination of Figure 3.

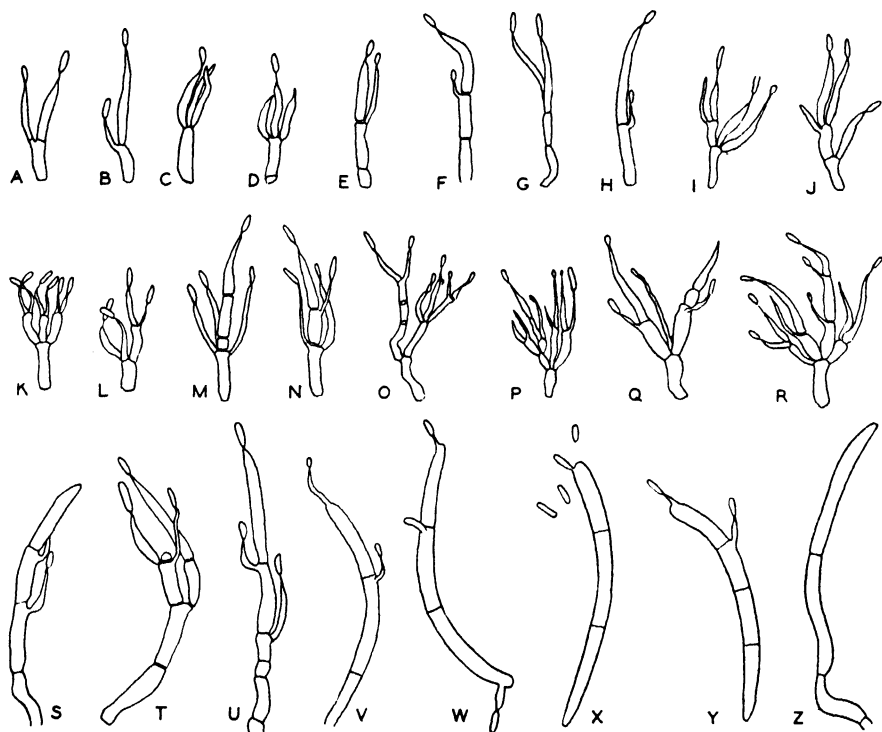


FIGURE 3. (A to R) Microconidiophores, showing variation in form, size, and complexity. (S to V) Abnormal microconidium-bearing structures with resemblances to macroconidia. (W to Y) Macroconidia producing microconidia. (Z) Macroconidiophore with macroconidium. A to Z, \times about 600.

A microconidium is borne at the tip of each branch. The number of microconidia produced by a single conidiophore is thus partly dependent upon the extent of the branching. The tip of a branch tapers to a sharp point and the microconidium originates as a small terminal swelling. A young microconidium is practically a spherical body. It increases in diameter and elongates to attain at maturity the form typical of these minute structures in this species (Figs. 3, 4, and 7 A). It contains a single

centrally located nucleus of large dimensions, considering the size of the cell; but it has not been possible to trace the passage of this nucleus into it through the thin neck at the tip of the conidiophore branch, or to observe the nuclear division which must take place in the terminal cell of the branch prior to the maturation of each microconidial element. There is evidence that several microconidia may be successively abstricted from the tip of each branch; hence the number of microconidia produced by a single much-branched conidiophore may be large.



FIGURE 4. (A) Microconidia and macroconidia taken from a single acervulus. (B) Microconidia from an infected leaf. (C) Microconidia and macroconidia produced in culture. A to C, \times about 525.

The number of microconidiophores present in an acervulus appears to be practically the same as the number of macroconidiophores present at an earlier date. Since the macroconidiophores are relatively short, simple structures (Fig. 3 Z), closely set on the thin stroma of the acervulus, it is obvious that when the relatively taller and complex microconidiophores appear in equal numbers, a region of extreme density results (Fig. 5 E). Just how the microconidiophores arise is not quite certain. First of all, it should be kept in mind that the changes occurring in an acervulus going over to microconidial production proceed rather gradually. That is, there is no point at which macroconidial production ceases suddenly; and no suggestion of disintegration of the old macroconidiophores was ever

seen. Since the macroconidiophores are closely set on the stroma of the acervulus, one cannot easily conceive of the microconidiophores growing out from the stroma as entirely new structures, and pushing up among the old conidiophores of the summer conidia. It may be that a few do so arise as branches from hyphae in the acervulus base, but such an origin for the whole mass does not agree with the observations made here. In cases such as those cited above, where purely microconidial fructifications are formed or where microconidiophores arise directly in the youngest part of a developing acervulus, they must take their origin directly from the stroma, but where macroconidia have been produced previous to the appearance of microconidiophores, we have quite a different situation. In the latter case it seems likely that the microconidiophores arise, at least for the most part, directly from the macroconidiophores already present. The structure of the microconidiophores is in keeping with such a conception of their origin. The basal cell not infrequently shows a close resemblance to an ordinary macroconidiophore (Fig. 3). On this, a branched superstructure instead of an ordinary summer conidium is formed. Occasionally lateral branches develop just below this superstructure. Certain microconidiophores show a definite "leader" trunk growing directly up from the basal cell, and resembling, except for its smaller size and lateral branches, a *Cylindrosporium* spore. Perhaps we are, in the case of such "leaders," dealing with what is morphologically equivalent to an aborted macroconidium. Moreover, in mounts prepared by dissecting in aceto-carmin an acervulus undergoing the transformation to microconidial production, microconidium-bearing structures may often be found (Fig. 3 S to V) which are larger than the average microconidiophore. These may show a striking resemblance to a macroconidium in general form, except that they usually possess some lateral outgrowths. They are obviously structures which started out to be macroconidia but which, when certain physiological conditions appeared in the particular leaf in question, became abortive, sent out branches, and proceeded to produce microconidia. This view is strengthened by the fact, easily verified, that normal, fully-developed macroconidia are potential producers of microconidia. Among the old macroconidia lying above the microconidial layer one may occasionally find, under certain circumstances, a *Cylindrosporium* spore which is either budding out microconidia directly or has sent out short protuberances at the tip or next to a septum, and at the end of these, microconidia may be seen to be forming (Fig. 3 W to Y). This is in line with the production of microconidia from macroconidia in *Sclerotinia* as shown by Woronin (70).

The microconidia in *Coccomyces hiemalis* are typically more or less rod-shaped (Figs. 4 and 7 A) averaging about $1.5 \times 4.5\mu$, although a range of 1.0 to 2.25μ in their diameter and 2 to 7μ in their length was observed.

There seems to be some tendency for the smaller microspores to be produced on the conidiophore branches that are of smallest caliber. Figure 4 A shows the contrast in size and shape of microconidia and macroconidia.

Although *Cylindrosporium* conidia can be produced in large numbers in cultures started either with ascospores or with conidia, microconidia are of rare occurrence in cultures. A few scattered ones frequently appear in cultures two or more months old, but they are sporadic. One single-ascospore strain which was obtained has, however, consistently formed these small bodies in relatively large numbers after growth for eight to ten weeks on lima bean agar. Figure 4 C shows macroconidia and microconidia taken from one of these cultures.

One of the features which Tulasne mentions as characterizing spermatia is their inability to germinate. That this criterion is inadequate is now clear. But the rareness of the occasions on which these bodies have been observed to put out germ tubes is remarkable. And the validity of at least some of the cases reported has not gone unquestioned. Long ago Möller (52) reported success in germinating lichen spermatia, but no one since has claimed to have induced germination of spermatial bodies in a lichen. Humphrey (47) described germination of the microspores of *Sclerotinia fructigena*. Brierley's account of microconidial germination in the case of *Botrytis* (22) was more convincing. Recently Dodge (35) has adequately demonstrated that in *Neurospora*, one of the ascomycete forms in which the functioning of the microconidia as fertilizers has been proved, these bodies will germinate to produce a normal mycelium. And within recent years some evidence of the ability of rust spermatia to germinate has also been adduced (2, 4, 6). Apparently these bodies are not completely lacking in capacity for vegetative development, but the fact remains that in general the spermatial bodies in both rusts and Ascomycetes do not germinate readily under the conditions under which the tests are run.

A number of germination tests were run using a variety of media and temperatures to see if germination of the microconidia of *Coccomyces* could not be induced. Microconidial suspensions of various concentrations were made by removing the microconidia from fresh leaves and placing them in sterile distilled water. Drops of the suspension were then spread on plates of a variety of standard agars. Tests were also run using cherry leaf decoction agar and the decoction itself. Incubation temperatures of from 5°C. to 30°C. were tried, but no signs of germination were seen during a period of four days, at the end of which time it was usually necessary to discontinue the experiment because of the growth of contaminants which could not be avoided on account of the source of the microspores. More extensive tests were run when the finding of the strain which produced microconidia in culture made it possible to have a source of material free from contaminating organisms. Drops of microconidial suspension on agar

plates and on clean sterile slides were incubated from 5 to 20 days without any indications of germination appearing. Higgins (45) also failed to obtain germination of these bodies.

STROMA FORMATION

The development of plectenchymatic stromata down in the leaf tissues in association with the acervuli, marks the first real step in the initiation of the ascocarpic stage in *Coccomyces hiemalis*.

About the last of August or the first of September when microconidia first begin to appear, stromata also soon begin to form, but at this time the development of microconidia usually precedes the development of stromata in individual infections. There is evidence, moreover, that microconidia may be produced for a very considerable period in some acervuli before there is any suggestion of stromata starting to organize below them. As the season advances, there is an increasing tendency for the two developments to begin together. In the case of infections taking place late in the season, it is not uncommon to see the situation which is typically found early, entirely reversed, stromata developing in connection with acervuli in which *Cylindrosporium* conidia are forming in abundance (Fig. 5 D). As in the case of the development of microconidiophores, the formation of the stromata seems to be conditioned by a complex of physiological factors.

It is interesting to compare the history of a midsummer infection with that of an infection developed as late as October. In the midsummer infection an acervulus naturally forms which, barring leaf-fall or the development of a "shot-hole" cicatrice which would remove the infected area from the leaf, will produce summer conidia whenever weather conditions are favorable, over a period of perhaps as much as two months. Then finally microconidiophores appear and a stroma develops, usually when the leaf begins to show signs of yellowing. In a late-season infection all these changes go on in surprisingly rapid sequence. Certain late infections induced by artificial inoculations were studied. An acervulus forms, but production of macroconidia may be limited to only a few days, or, as we have seen, microconidiophores may appear in the acervulus even before it has completed its lateral growth. Likewise, stromata may begin to be organized in a remarkably short time after the fungus has effected entrance. Stromata of considerable size were observed in some of these spots less than ten days after inoculation. In one experiment where small experimental trees with healthy green foliage were inoculated late and infection obtained was heavy, the leaves started to yellow as soon as the spots showed up, stromata appeared early; and twelve days after inoculation all the leaves were off the trees. Subsequent examination of the fallen

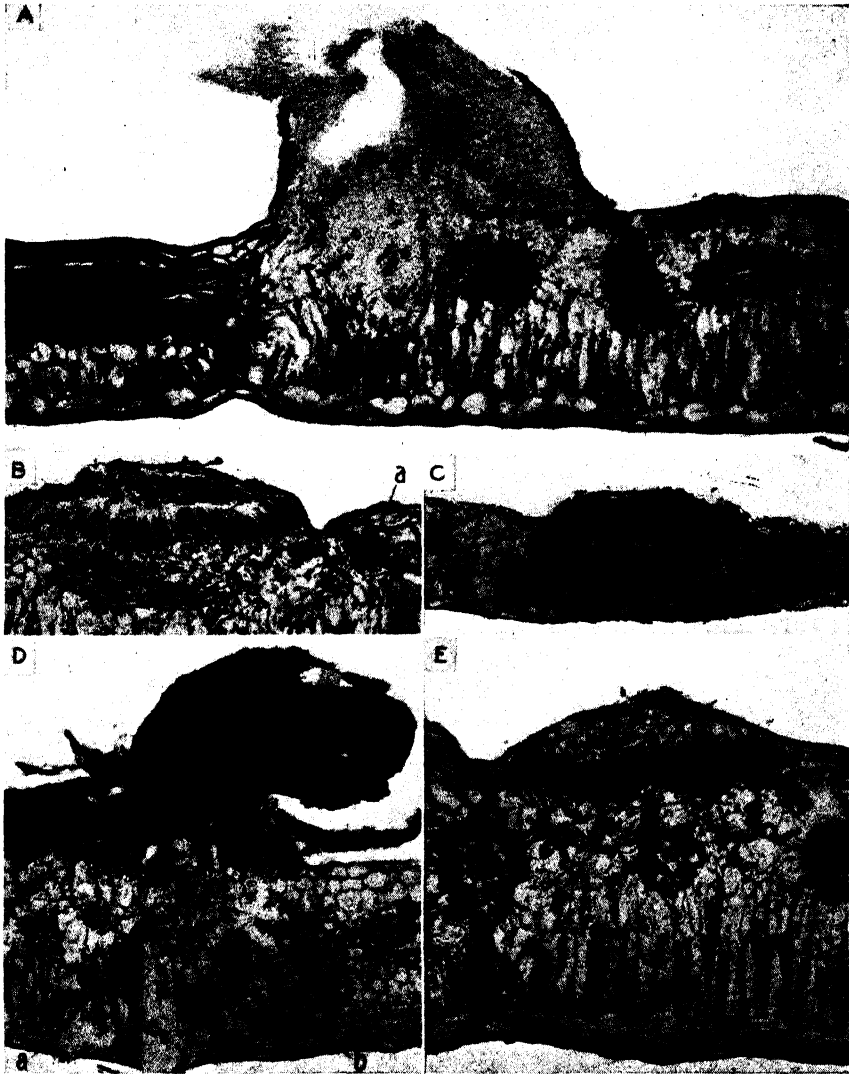


FIGURE 5. (A) Cross-section through leaf, showing typical situation with reference to ascocarp initiation. Microconidial mass forcing up the epidermis; macroconidia visible in uppermost region of acervulus above spermatia; stroma in contact with acervulus base; coils clearly discernible. \times about 115. (B) Section showing young stromatal patch (at *a*) laterally displaced with relation to associated acervulus. \times about 105. (C) One large and one small (younger) stromatal patch beneath acervulus. \times about 69. (D) Young stromatal patches (at *a* and *b*) formed deep in mesophyll below acervulus producing macroconidia, from a relatively young infection produced in late October. Section median for stromatal patches. \times about 105. (E) Microconidial acervulus. No stroma yet formed. \times about 155.

leaves showed that the normal changes leading to passage of the fungus into dormant condition for the winter were occurring in them.

The stromata commonly arise as what may be termed "stromatal patches." From Higgins' brief account of stroma development (45) one cannot get an adequate picture of this interesting situation.

When conditions are favorable for stromata to form, there develop down in the mesophyll, usually immediately below the acervulus base, one or more compact masses of much-branched, fairly closely interwoven hyphae. More or less spherical in contour, as a rule, and usually with a rather well-defined border from almost the first, these plectenchymatic masses stand out as prominent reddish areas in free-hand sections stained with erythrosin or in microtome sections delicately stained with Heidenhain's haematoxylin and counterstained with erythrosin. The size shown by patches just organized is various. Some are extremely minute—not more than 30μ in diameter, while others are much more extensive, but in no case, except when formed beneath a very small acervulus, do they have a diameter which approaches that of the associated acervulus (Fig. 5 B to D; Fig. 8 A, B, and C).

In the initiation of a stromatal patch the hyphae may mass together to form a single small compact "nest" at inception, or a larger area may be described by an only moderately dense growth which is sometimes more or less sponge-like in character and with more or less indefinite limits, and then later it becomes compacted; still further, two or more minute initials may develop close together (Fig. 1 B), sometimes entirely separate, and sometimes more or less connected, but in all cases apparently fusing together very early to form a single patch.

As previously mentioned, usually the stromatal patch forms immediately below the acervulus base—often practically effecting a union with it at the time of its formation (Fig. 8 B and E). In other instances it can be seen that although the incipient stroma lies very near the base of the conidial fructification, actually it is not in contact with it (Fig. 1 B, 2 B, and 8 A), and here it is clear, then, that the patch has arisen from the mycelium as a separate entity. One who studies large numbers of slides can find, too, a considerable number of cases where a stroma has taken its origin deep in the mesophyll (Fig. 5 C and D; Fig. 8 C). It may arise in an isolated position at the level where the palisade cells abut upon the spongy parenchyma or even near the upper epidermis, at the extreme opposite side of the leaf from the acervulus, or still further may, on rare occasion, show lateral displacement (Fig. 5 B).

At this point it may be mentioned that occasionally the stromatal patch phenomenon is not well defined. Instead, the stroma may begin as a broad or irregular and sometimes poorly delimited plectenchymatic growth beneath the acervulus, but this situation is unusual. In the large

majority of cases the development proceeds along such lines that the incipient stroma may be identified as a definite spherical or ovoid patch.

Most typically a single stromatal patch develops in association with each acervulus (Fig. 5 A), and if the conidial fructification is small, no more than one ever forms, but when the acervulus is of large dimensions, occasionally two or three plectenchymatic masses may be organized (Fig. 5 C and D; Fig. 8 A to C). In the case of certain very large acervuli which may range from 600 to 700 μ in diameter, four, five, or even more associated stromata may sometimes be counted, but here the situation is complex, for it is doubtful if such large acervuli are often simple ones. There is good evidence to show that they frequently result from fusion of several smaller ones which grow together as they expand laterally. Each stromatal patch is potentially the fundament of an ascocarp, and consequently one may expect to find the number of ascocarps on a given leaf exceeding the number of acervuli present the fall before. As a matter of fact, when one examines the surface of overwintered leaves with a dissecting microscope, he frequently finds small groups of closely associated ascocarps covering an area equivalent to that occupied the preceding year by a large acervulus and the immediately associated mycelium.

But although each stromatal patch represents fundamentally the initial of an ascus fruiting body, study of their further development shows that the number of young patches which are found may not be an entirely accurate index of the number of apothecia which will be developed. The plectenchymatic mass, which typically forms directly below the acervulus base, increases in size by peripheral growth; and, if it was not originally in contact with the acervulus stroma, soon effects such contact, and thenceforward its chief expansion is downward and lateral. Very commonly in this species of *Coccomyces* a stroma comes to extend through the entire leaf to the opposite epidermis (Fig. 2 D; Fig. 8 D, F, and G). Meanwhile it has increased proportionally in width. In the case of the stromata taking their origin in a position relatively remote from the acervulus base, it seems clear that most often these too eventually effect contact with the acervulus through peripheral expansion. The exact fate of certain of the laterally displaced ones is not clear, however. When two or more stromatal patches are formed beneath a single acervulus, these may, as they expand, come into contact and then fuse—especially if they arose near one another. A single large stroma is the product, and from this a single large ascocarp results. Other types of fusions occur. Sometimes, as we have seen, in a single infection an acervulus is developed at both the lower and upper surfaces of the leaf (Fig. 8 D). A stroma initial ordinarily develops below each acervulus, and as these initials expand they commonly meet and unite to form a single large stroma extending through the leaf. A "double ascocarp," i.e., an ascocarp possessing one stromatic base but producing

asci at both leaf surfaces, often results from the product of a union of this kind. Many examples of imperfect fusion may also be seen. Certain stromata come into contact laterally only when nearly full-grown. In the spring the hymenia derived from these stromata usually are continuous with one another but maintain their individuality except at the point or points of contact. Below the surface of the leaf such a fructification may show two or more almost distinct stromatic bases or a single fusion base with well defined lobes corresponding to the stromata which entered into the fusion.

Considerable variation exists in the size attained by stromata. Many extend through from epidermis to epidermis. Others remain small. A stroma organized beneath a small acervulus often remains shallow, and sometimes never attains a diameter of more than 70μ . Such give rise to small apothecia with thin stromatic bases. We have seen how fusions of stromatal patches account for the large size attained in certain instances; and one can observe certain correlations between diameter of the acervuli and the size of associated stromata, but most of the factors involved in the determination of stroma magnitude remain unknown.

ARCHICARP DEVELOPMENT

Within the stromata coiled archicarps appear in considerable numbers. Higgins (45) has stated that six to eight may be present, but apparently he has underestimated the number that may occur. A single large stroma may show as many as a dozen or even more. It has not been possible to ascertain just when the first coils arise because at first they do not stain differentially and it is not easy to distinguish them. They were observed at times, however, in very young patches. They do not all appear at one time, but as the stroma increases in size new ones keep appearing, especially in the peripheral regions. It is clear that ordinarily the coils form at various points within the organized stroma, but occasionally their formation just outside of a growing stroma at its periphery was observed. In such a case, however, they are soon enclosed by the hyphae of the expanding edge of the plectenchymatic mass.

The coils develop in practically all portions of the stroma (Figs. 2 D and 5 A), and sections reveal them oriented in various planes. Each coil is made up of from eight to a dozen uninucleate cells (Fig. 7 D) and is a more or less helical structure of two to three turns (Figs. 2 E, 7 B, C, and D). The cells making up the coil are not of entirely uniform size. They tend to be of somewhat smaller diameter at the distal end, but there is no such contrast in size as in some other Ascomycetes—*Ascobolus*, for example. The coil cells may be slightly larger or slightly smaller than the surrounding cells of the stroma, depending upon the circumstances.

In microtome sections these helical structures are usually cut into sev-

eral pieces, and it is only in a few favorable thick sections that it has been possible to trace the windings of a coil in such slides (Fig. 7 D), but these archicarpic structures, despite their small size, can be dissected out of the stromata when hand-sections of stromata are stained in aceto-carmine, and advantage was taken of this technique in studying the gross anatomical features of the coils. Figure 7 B and C shows sketches of coils dissected out in the fashion described. A coil seems to grow out as a lateral branch of a stroma hypha or, in such cases as were mentioned where the coil originates outside the stroma, from what appears to be an ordinary vegetative hypha. The coiled basal portion of the archicarp develops first; but eventually each coil is prolonged by a multicellular trichogyne which usually winds more or less tortuously upward, grows through the base of the microconidial acervulus, and ends somewhere among the microconidia (Figs. 2 E, 6 A to D). It is certain that in at least some cases a considerable period elapses after the coil itself has taken form, before the trichogyne develops.

Ordinarily a trichogyne does not pass outside of the stroma until it reaches the acervulus, but in certain instances where a stromatal patch took its origin near the upper epidermis and failed in its growth to meet the acervulus base, or in certain cases where the stroma showed lateral displacement with relation to the microconidial fruiting body, the trichogyne could occasionally be traced passing out of the stroma and growing across a region where no stromatal hyphae were present, to reach the acervulus. The trichogynes, regardless of the position in the stroma of the coils to which they belong, show a decided tendency to grow to the region where microconidia are being formed, but they do not always take a direct path to reach that region. They frequently grow out at various angles. It may be that certain portions of the stroma prove too dense to allow growth directly upwards, and the trichogyne takes the path of least resistance; or it may be that the tropic stimulus or response is not sufficiently strong at first to insure attainment of the final position by the shortest possible route. Exceptionally a trichogyne may fail to grow in the direction of the acervulus at all. Cases have been seen, especially where the trichogyne prolonged a coil lying in the upper half of the leaf, in which this structure was growing directly towards the upper epidermis where no acervulus was present. No evidence has been uncovered to show that such trichogynes ever reach the exterior of the leaf. The length of a trichogyne and the number of cells in it are variable and depend, at least in part, upon the position of the coil to which it belongs. It is obvious that in the case of a coil formed immediately below an acervulus base, the trichogyne will likely be much shorter than where a coil develops in a position remote from the acervulus.

The trichogynes appear to develop comparatively rapidly once they

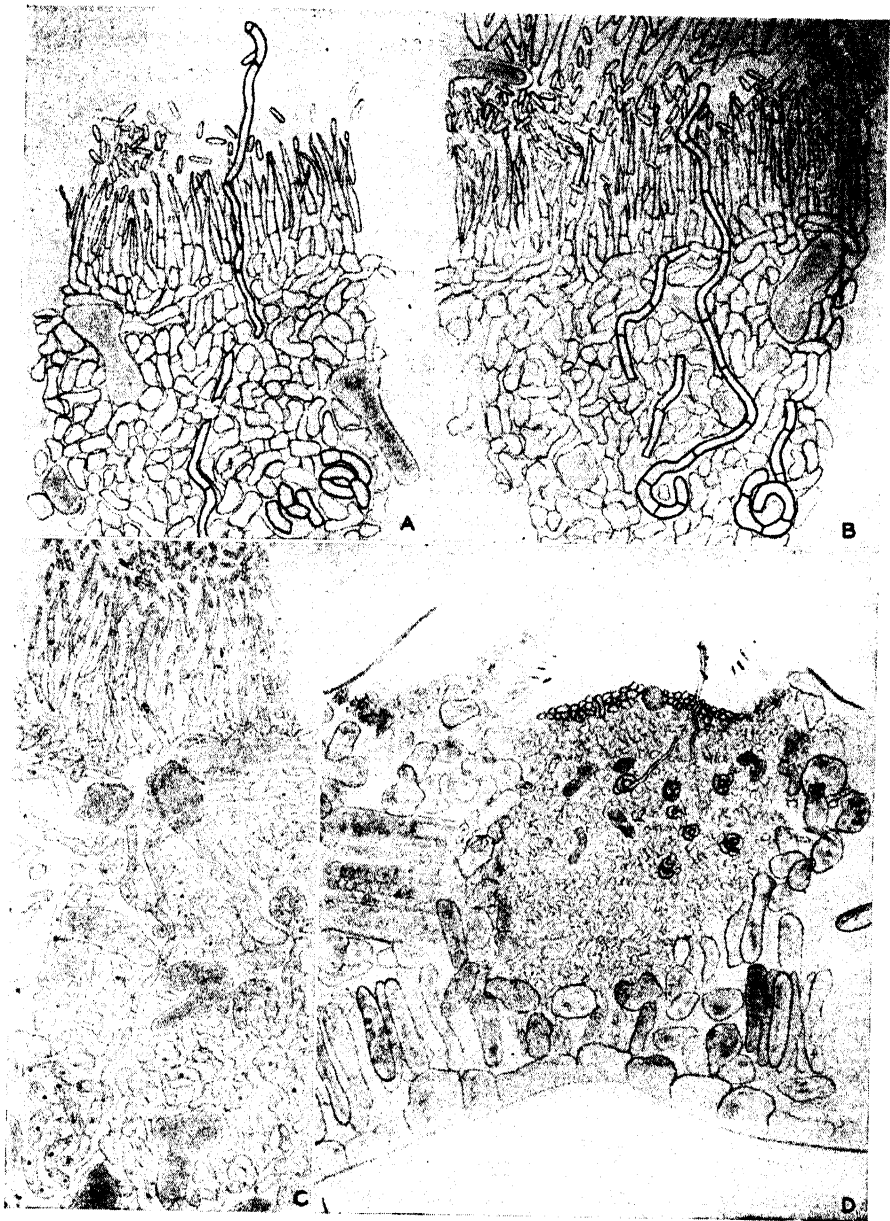


FIGURE 6. (A, B, and C). (From microtome sections.) Sketches showing trichogynes projecting into acervulus region. \times about 650. (D) (From free-hand section.) Microconidia attached to end of trichogyne which can be traced down into stroma. Several coils discernible in stroma. Most of microconidiophores torn away in making preparation. \times about 220.

start to grow, and they are relatively ephemeral structures; at least their upper portions are short-lived. One finds the maximum number of them in a healthy state in leaves collected from the tree a short time prior to leaf-fall, in yellowing or green leaves, depending upon the extent of infection and the advancement of the season. Even before the leaves drop, the upper portions of many of the trichogynes disintegrate; and not uncommonly one finds sections where stromata are full of coils and very healthy-looking lower portions of trichogynes, yet no trace of a trichogyne can be found in the acervulus region.

In microtome sections of imbedded material it is extremely difficult to trace a trichogyne from its origin at the coil to its tip in the acervulus region. Because of the various angles at which the trichogynous structures grow and because of the tortuous path they may follow, they are almost always cut into several sections. To trace them with certainty from section to section in the dense stroma is very rarely possible, especially when there is a large number of them present to cause confusion. Their small size, and the uncertain differential staining which they may show, add to the difficulties, as does also the fact that in a given stroma the upper portions of many trichogynes are likely to have already degenerated and hence these trichogynes end blindly or taper off indistinctly somewhere in the tangled mass of stromatic hyphae through which they extend. As for following up any trichogyne and studying its terminal parts in the acervulus region, one is hampered here by even more obstacles. It is well to keep in mind in this connection a picture of the acervulus region at this stage; the general features of it are illustrated in Figure 2 B and D and Figure 5 A. It is a region of great complexity. The density of the microconidiophore layer has already been mentioned. Above the conidiophores lie the microconidia in immense numbers, and usually some old macroconidia can still be found lying above these. The situation may be further complicated by the belated development of an occasional macroconidium among the microconidiophores. A portion of such a summer spore cut in the sectioning may strikingly resemble a trichogyne fragment. Foreign hyphae of one sort or another also occasionally penetrate this region and may cause confusion. These may be stray branches from the mycelium of some saprophytic organism growing on the surface of the leaf, or more rarely are germ tubes from old macroconidia (under favorable moisture conditions a few of these spores may sometimes germinate in the upper portions of the acervulus at this stage). Sections thick enough to enable one to trace the trichogyne adequately, are entirely too thick to allow one to decipher any possible trichogyne-microconidium relations. Figure 6 A, B, and C shows sketches made from sections of fixed material where trichogynes could be traced up into the acervulus region. According to Higgins, the trichogynes end just above the microconidiophores and are

swollen at the tip. Often, it is true, they do end in the position indicated, and, as Figure 6 C shows, they may be swollen at the tip, but it has many times been possible to trace them growing up much higher into the microconidial region, and they are not always particularly enlarged at the end.

TRICHOGYNE-MICROCONIDIUM RELATIONS

Because of the unsatisfactory opportunity to study possible relations of the microconidia to the terminal portions of the trichogyne afforded by microtome sections of fixed material, and in view of the vital importance of investigating this particular point, other means of getting at the situation were sought. It was found that free-hand sections provided the best way to approach this problem; and although they left much to be desired, yet a study of them has revealed several things of interest and importance. The first preparations of this kind were made in the fall of 1931, but the scarcity of fresh material at that time prevented accomplishing more than working out the technique and ascertaining the possibilities of the method. During October, 1932, a few days were spent in the mycological laboratory at Cornell University in preparing and studying sections of infected leaves obtained fresh from the University orchard there; and although some interesting clues were obtained at that time, nothing conclusive was found. In the fall of 1933, however, there was available throughout September, October, and the first part of November a most satisfactory supply of diseased leaves, as the result of inoculation of a large number of small potted trees, and this made it possible to prepare a relatively large number of slides for study.

Small pieces containing "spots" were cut from yellowing or green leaves recently removed from diseased trees, care being taken to select such specimens as experience gained in study of the fixed material had taught would be most likely to show trichogynes in their prime. Sections were then cut through the spots and stained with erythrosin, aceto-carmin, or cotton-blue, the first stain mentioned being employed chiefly, however, as it yielded the most satisfactory results. From among the sections cut, those which were thinnest and also showed well-developed stromata were then selected with the aid of a binocular dissecting microscope, and after the excess stain had been washed out, were transferred to a drop of water, alcohol, or glycerine on a new slide. Examination of such sections at this stage of preparation usually shows the acervulus region presenting as much complexity as one finds in slides of microtome sections. The layer of microconidiophores is discernible, and above them a maze of microconidia grading off into a mass of the old persisting macroconidia in the uppermost region. In this situation it is usually quite impossible to see whether any trichogynes are projecting into the acervulus region, but the advantage of this type of preparation lies in the fact that

an opportunity is afforded to simplify the situation. By exerting pressure on the cover glass and by other manipulations, by transferring the sections from drop to drop of the mounting fluid, etc., one can frequently succeed in floating away the microconidia and the macroconidia and leaving the microconidiophore layer directly exposed. There can be no doubt that the delicate ends of the trichogynes are at least occasionally broken off and floated away with the other material, but such as do remain intact can be studied advantageously. It should be remembered, though, that the chances, first, of finding a spot where there are trichogynes in prime condition, then of obtaining a sufficiently thin section and bringing this through all the manipulations indicated, without destroying the essential elements, are not large; and much work is involved in obtaining a few slides.

In studying these preparations the chief object of course was to discover whether microconidia ever become attached to trichogyne ends, in the manner Stahl described in the lichens. In view of the organization of this fungus, as revealed by the morphological and cytological studies thus far made, one is led to believe that if an act of fertilization is an essential feature at this point in the life cycle of the species, it must involve passage of a nucleus or nuclei into the coil by way of the trichogyne, and the situation is such as to suggest the microconidia as the most logical source of such nuclei. Attachment of microconidia to trichogyne ends would in turn appear to be the most logical way of effecting that intimate contact which would allow transfer of a nucleus from the one structure to the other.

Early in the course of the examinations of the free-hand sections this past fall (1933), there was observed in an occasional acervulus a structure which it was suspected represented the terminal portion of a trichogyne, apparently growing up from among the microconidiophores and sometimes extending quite high above them (Fig. 8 E). Moreover, it was seen distinctly that microconidia were attached to the majority of these structures. Sometimes, in fact, a most spectacular appearance was presented, microconidia in large numbers clinging around a single one of them (Fig. 7 E). It must be said that in the latter cases further tapping and pressure on the cover glass would dislodge a major portion of these. However, the fact that they had previous to this been subjected to sufficient manipulation to remove all free structures from the field, is of interest since it would indicate that some substance was present—possibly a product of the structure around which the microconidia were massed—which acted to retain the minute bodies. Although, as was stated, the majority of the microspores in a situation like that pictured in Figure 7 E, can be eventually removed, a few almost always resist all efforts to get rid of them. Actually the structures which it was suspected were trichogynes were only rarely so thoroughly loaded as in the cases just mentioned; most often

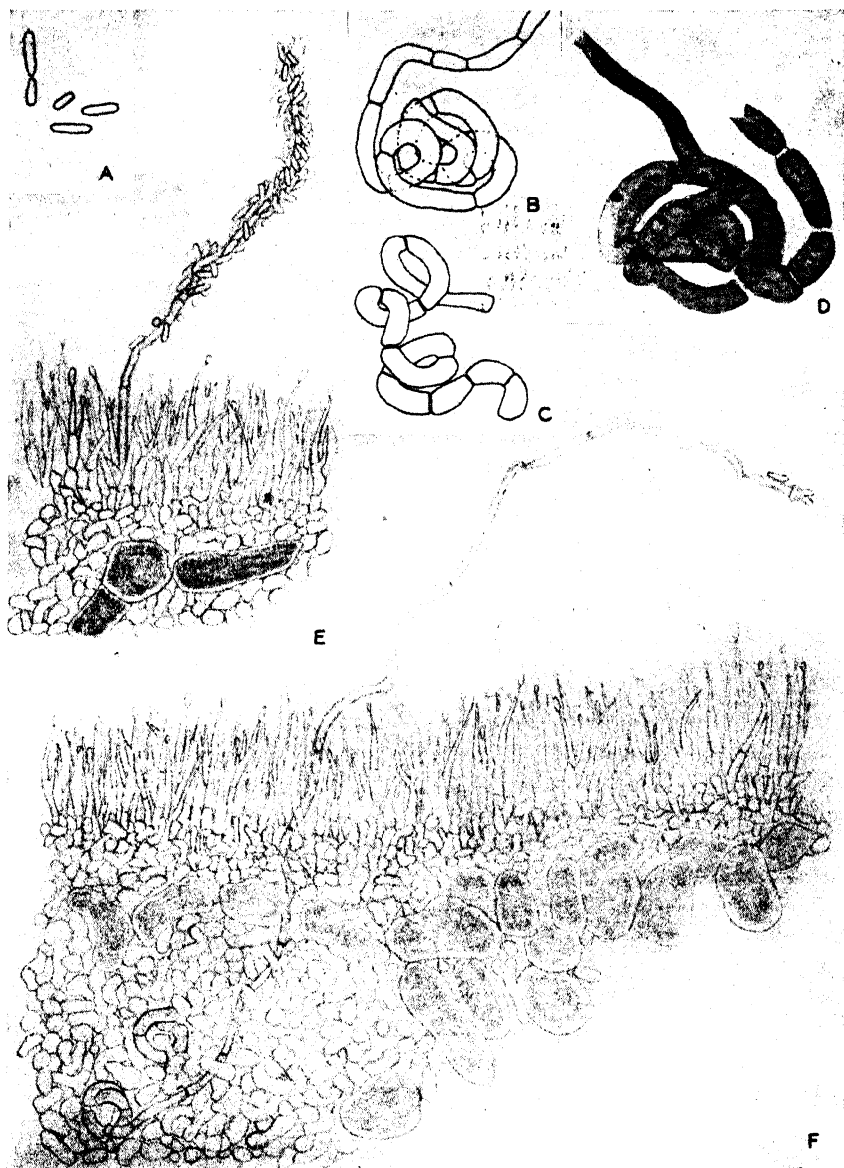


FIGURE 7. (A) Microconidia. \times about 900. (B and C) Sketches of coils dissected out of stromata stained with aceto-carmin. Method of coiling illustrated. In C coil stretched out slightly to show number of cells, etc. \times about 900. (D) Coil from cytological preparation. \times about 1450. (E) From stained free-hand section through acervulus. Upper end of trichogyne projecting above microconidiophore layer and loaded with adherent microconidia. Free microconidia and macroconidia removed as described in text. (F) Free-hand section through portion of an acervulus and stroma, showing trichogyne which can be traced from its origin at coil to tip high above microconidiophores. Microconidia attached to tip of trichogyne. Free microconidia and old macroconidia removed as in E so as to expose microconidiophore layer. E and F, \times about 650.

only from two to seven microconidia were found adherent, but these usually proved to be most tenacious.

As stated above, it was surmised that these microconidium-retaining structures were the upper portions of trichogynous hyphae. The fact that they presented a picture identical with what one would naturally expect on the basis of study of microtome sections, the fact that they extended out from among the microconidiophores, the occurrence of two or three of them in a single acervulus in some cases, and their presence only in parts of the acervuli below which a stroma was found, still further their staining, corresponding quite well with the staining of the coils discernible in the stromata below, all indicated that these were in reality trichogynous structures. However, the fact that hyphae of saprophytic organisms, and germ tubes derived from old macroconidia which occasionally germinate above the microconidial mass have been found on occasion penetrating into the lower parts of the acervulus, also the fact that it is conceivable that the stroma hyphae might give off stray branches which could grow up to the surface and into the acervulus region (although no evidence of such a situation has been seen in microtome sections), all these things make caution necessary, but I was able for the most part to adequately satisfy myself that the hyphae to which the microconidia were attached were not of such nature or origin. Admittedly, however, the best proof of the trichogynous character of these hyphae would be to demonstrate that they were connected to coils. Unfortunately it is ordinarily extremely difficult to identify a trichogyne down in the stroma in the type of preparation used. Early in the investigation, however, there were obtained some sections like that shown in the sketch in Figure 6 D, where it was possible to follow the hyphal structures with attached spermatia, down into the stroma tissue for a short distance. Also the proximal portions of trichogynes could be traced running upward from the coils. Finally, in a few very thin favorable sections it was found possible to trace the trichogyne from its origin at the coil to its tip far outside the stroma. The coil shown in Figure 7 F was located near the surface, hence the possibility for the trichogyne to escape being cut. Differential staining of the whole archicarpic system, emphasized by the thinness of the section, made it easy to follow the trichogyne throughout its total length of about 250μ . It is composed of nine or ten cells and increases somewhat in diameter after passing outside of the stroma. To its tip high above the microconidiophore layer four microconidia were found attached, and so firmly attached that such rigorous treatment as threatened to disintegrate the whole section, failed to dislodge them. From these observations it seems clear that in *Coccomyces hiemalis* microconidia do become firmly attached to trichogyne ends, but as yet it has not been possible to observe any actual fusion between the spermatial bodies and the trichogynous hypha. No opening could be found

between the two structures such as Stahl (63) and Baur (14) have figured in the lichens and Allen (6) and Craigie (30) have pictured in the case of the attachment of spermatia to receptive structures in the rust fungi, nor have any good indications of gelatinization of the cross-walls of the trichogynes with microconidia attached, been seen. Certain observations of a cytological character have been made, however, which would suggest the presence in the trichogyne cells of extraneous nuclei possibly of spermatial origin, but I should not care at this time to press this forward as significant evidence that spermatial nuclei enter the trichogyne.

MORPHOLOGICAL CHANGES FOLLOWING LEAF-FALL

Among the phenomena associated with ascocarp initiation in this fungus there remain to be considered the changes in the stroma preparatory to overwintering, and the development of the saprophytic mycelium. As Higgins (45) has already pointed out, prior to passage of the fungus into a resting condition for the winter, the outermost portion of the plectenchymatic stroma becomes modified to form a pseudoparenchymatous covering or rind, the cells composing which, become thick-walled and dark in color (Fig. 8 F and G). This develops shortly after leaf-fall. Infected leaves that dropped in early November in Yonkers, New York, showed the covering well along in its development within two weeks after the leaves were on the ground. While the pseudoparenchymatous rind is being organized, the microconidiophores at the surface of the leaf begin to disintegrate and gelatinize. Soon whatever remains of the acervulus is completely separated from the stroma plectenchyma below by the thick-walled rind. Associated with these changes at the exterior, changes also go on within the stroma. More or less complete disintegration of the trichogynes ordinarily ensues, although, curiously enough, persistent trichogyne segments may occasionally be identified in sections of material fixed after the leaves have been on the ground many weeks. The differentiation of the coils is accomplished with continually increased difficulty as the organism passes into resting condition. They were not visible with certainty in dissected material later than the middle of December; and in fixed material the difficulty of getting fixatives to penetrate the pseudoparenchymatous covering of the stroma, and the reduction in the possibility of obtaining sharp differentiation of the ascogonial structures by staining, consequent to changes in the protoplasm of their cells passing at this time into a state of rest, make accurate observations in this direction difficult at late dates. The fate of the coils will be considered, however, in another paper in which it is proposed to present a cytological study of the development of the ascogenous system.

With the death of the leaf tissues at leaf-fall, the fungus passes from a parasitic existence to one of saprophytic relationships. Correlated with

this, marked changes occur in the mycelium. Even before leaf-fall, in yellowing leaves, modifications in the hyphae may be seen, which presage the development of the saprophytic mycelium. The chief modification at this time is the formation of very conspicuous vacuoles in the cells of the hyphae. Once the leaves have dropped, more drastic changes promptly ensue. While, in the simplest cases, the saprophytic mycelium appears to result for the most part from mere transformation of the mycelial threads already present, in other cases rather extensive new growth is also involved. Branches grow out from the hyphae at various points, and in places dense mats of new saprophytic filaments appear, particularly in the vicinity of the stroma. Development is strongest in the spongy parenchyma, but strands also frequently push down in abundance between the palisade cells (Fig. 8 G and I); the dead host cells may sometimes be crushed (Fig. 8 H). The new mycelium may also extend out somewhat beyond the limits of the parasitic growth, but such extension is always limited. The cells of the new hyphae are very different in appearance from those of a hypha in a summer infection. They are shorter, in general, thicker, and tend to be somewhat more irregular. The cells of the old, formerly parasitic mycelium which was present prior to leaf-fall, also, as they take up a saprophytic life, undergo further changes, sometimes dividing and becoming modified in shape. Finally, with the approach of cold weather, the cells of all the hyphae present become thick-walled, and the walls assume a brownish tint. The nucleus in these cells does not lose its sharpness as a result of this modification, however. All these transformations of the mycelium result in a picture (Fig. 8 G to I) which contrasts sharply with that one finds while the leaf bearing the spot is still on the tree.

NOTE ON CERTAIN UNUSUAL ENVIRONMENTAL EFFECTS

It was pointed out earlier in this paper that ascocarp initials are not formed in connection with every spot developed during the growing season, there being indications that, especially among the earlier infections of the season, many spots are in one way or another removed from the tree prior to the occurrence of proper physiological conditions favoring the development of stromata and microconidia. It seems worthwhile to record in addition certain observations which show how development of ascocarps may occasionally be prevented in quite a different way. During the fall of 1930 a small private cherry orchard at Madison, Wisconsin, was frequently visited. The trees showed only moderate infection, and most of the spots were of relatively late origin. Few of the leaves yellowed prematurely. In early October a considerable number of acervuli showed only macroconidia present. Formation of ascocarp initials was relatively retarded. Shortly thereafter there came suddenly a freezing period that was unusually early and of unusual severity and duration. All of the cherry leaves

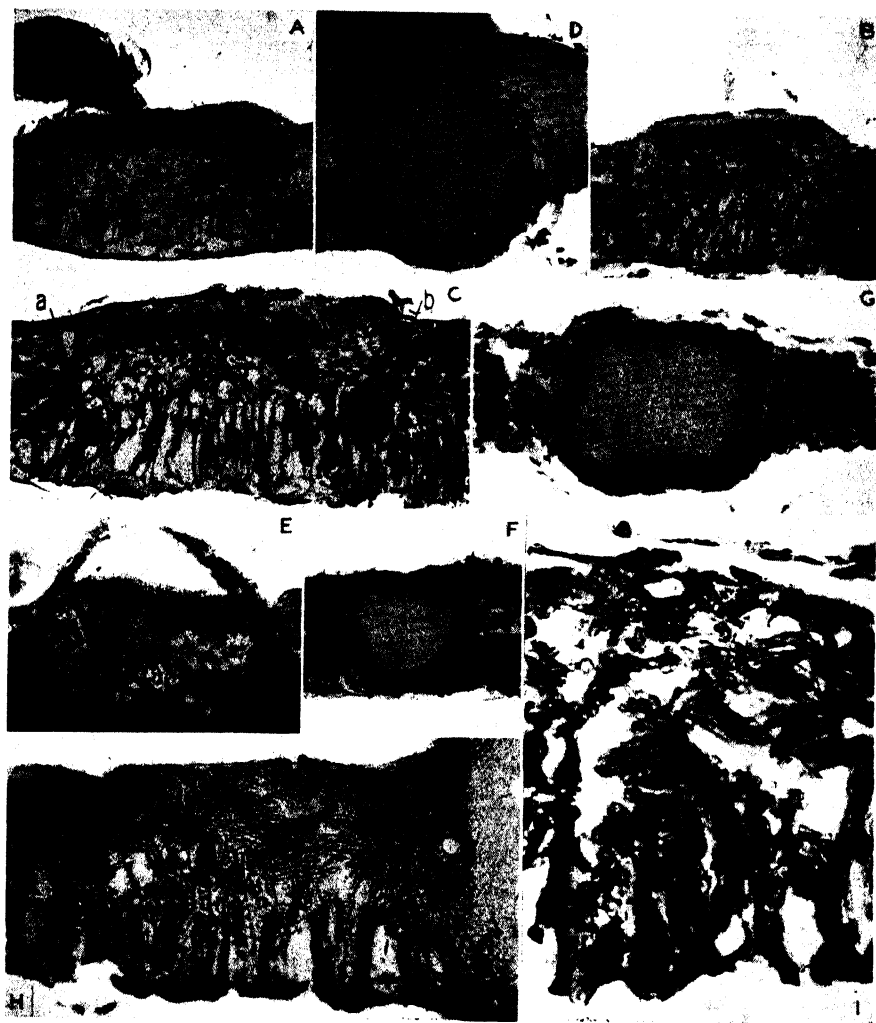


FIGURE 8. (A, B, and C) Stomatal patch phenomena. In B young patches in contact with acervulus base. In C two widely separated young patches (*a* and *b*) deep in leaf tissues. B, \times about 64; C, \times about 97. (D) Stroma extending from epidermis to epidermis. Acervulus at both surfaces of leaf. \times about 97. (E) Free-hand section through acervulus, showing trichogynes projecting, after removal of conidia and spermatia from region. \times about 97. (F and G) Sections through leaves showing stomata in resting winter condition. G, \times about 97. (H) Section showing thick-walled saprophytic mycelium. Cf. mycelium of parasitic stage in Figure 1 A. (I) Section showing detail of saprophytic mycelium. \times about 354.

were frozen and hung on the tree dry, brittle, and green, for an entire week, until warmer weather returned, when they turned brown and fell off. A quantity of these leaves was picked up and put in an isolated spot to overwinter. Casual examination from time to time later showed that few of the normal developments were occurring even in the case of spots where stromata had begun to form prior to the freezing. In some cases the mycelium appeared to be dead. No ascocarps matured on any of these leaves the following spring, although a few scattered fructifications bearing apothecial conidia developed.

The weather conditions of the fall of 1930 at Madison, as detailed above, were very abnormal; and it is doubtful if low temperatures often thus become an important factor in upsetting the normal course of events in the development of this organism, at least in the case of a sufficient number of leaves or spots to reduce significantly the amount of ascospore inoculum available the next spring to establish the leaf-spot disease on the new foliage. From the standpoint, then, of the disease cycle, the phenomena recorded are probably unimportant, at least in the locality where these observations were made, but the observations are of interest in pathology in relation to the question of the physiology of *Coccomyces*, and, in particular, relative to the problem of the environmental factors which may play a part in conditioning, modifying, or inhibiting the initiation and development of the perfect stage.

DISCUSSION

Several types of spermatial or microconidial bodies have been described in the Ascomycetes. The short bacilliform type, of which the microconidia of *Coccomyces hiemalis* are fairly typical, is probably the most common. Bacilliform spermatia are also found in a large number of the Ascolichens. A second common type is the globular form of which those found in *Sclerotinia* are characteristic. It may be noted that in *Sclerotinia gladioli*, *Neurospora*, and *Pleuraea anserina*—the only cases where the ability of the microspores to effect fertilization has been demonstrated—these bodies are spherical or at least more or less so. In the Laboulbeniales both spherical and short bacilliform spermatia are known, and Thaxter (64) has figured copulations of both types, with trichogynes. Among the red algae, likewise, both spherical and bacilliform spermatia are known; and in the figures in Bornet and Thuret's classic work on fertilization in the Florideae (19) both types are shown fused with the ends of trichogynes. The resemblance of these algal spermatia to the microspore structures of the Ascomycetes just described, and the similarity of the copulation pictures in the two groups, are striking. Besides the two types of microspores already mentioned as occurring commonly in the Ascomycetes, still others have been described. *Polystigma rubrum* (17, 51) forms small elongated

bodies which do not germinate; and small bodies, thin, but extremely elongated, have been figured for *Gnomonia erythrostoma* (23, 51) and *Hendersonia foliorum* (51). These elongated bodies have been referred to as spermatia at various places in the literature, although some have preferred to term them microconidia or simply degenerate conidia. That all these cells, like the spermatia or pycnosporos of the rusts, possess, however, characters which we ordinarily associate with male sex cells—reduced amount of cytoplasm and a proportionally large nucleus, all admit.

We have seen that the summer conidia of *Coccomyces hiemalis* under certain circumstances produce small cells which at least morphologically are indistinguishable from the microconidia produced on microconidiophores. I have observed the same phenomenon in the case of another phacidaceous form, *Diplocarpon rosae*. Microconidia formed by budding and otherwise on ascospores in species of *Dermatea* have been described and figured by Bayliss Elliott and Chance (15), Gregor (40), and Boudier (20, v. 3 pl. 559, v. 4 p. 330). Spectacular examples of the formation of microconidia on ascospores were long ago described and figured by Woronin (70) in the case of species of *Sclerotinia*. Drayton (37) was able to make similar observations on *Sclerotinia gladioli*, and he succeeded in demonstrating here that these microconidia are as capable of effecting fertilization as those produced in ordinary fashion.

De Bary (13, p. 262 ff.) classified under the caption of "zweifelhaften Spermatien" ("doubtful spermatia") certain spermatiform bodies produced in various Ascomycetes, which in all their characters seemed to him to answer the description of spermatia, but instead of being produced in or on distinct spermogonia, were seen to be abscised at a variety of other points in the thallus—from the mycelium and germ tubes, from germinating spores directly, from paraphyses, in pycnidia producing also regular spores, etc.—and thus to show in the time of their formation no such definite relation to the time of ascocarp initiation as is seen in the case of the spermatia in the lichen fungi, for example. De Bary included under this head the microconidia of *Sclerotinia* which, thanks to Drayton, we now know are capable of bringing about fertilization in at least one species of the genus. In several species of Ascomycetes small bodies which show the general morphological characters of spermatia and which either refuse to germinate or are germinated only with difficulty, have been described by various authors as "B-spores," etc., and are by many regarded as reduced conidia. It is an open question whether in reality these are conidial in nature or should rather be regarded as showing closer affinity with true spermatial elements. Lack of knowledge of the cytological characters in many cases and lack of adequate tests of function of these bodies naturally hinder proper classification. However, function alone is a poor criterion in working out homologies, and it is especially unsatisfactory in

the fungi where plasticity and reductions are everywhere in evidence.

Whether the microconidial or spermatial bodies of the Ascomycetes are fundamentally the same as the spermatia in the red algae or have been derived from conidia by reduction is a question which has long been argued by mycologists interested in the implications of the problem from the standpoint of evolution. The definite proof recently brought forth, of the functioning of the microconidia as fertilizing agents in certain forms, naturally brings renewed interest to the question and it also brings new problems, since if one supposes these bodies to be of conidial origin, then their present rôle as fertilizers involves the introduction of a most interesting substitute process.

In the case of the large majority of Ascomycetes where microconidia are known, these bodies are ordinarily abstricted from the ends of "conidiophores," as in *Coccomyces*—a method of origin not so remote from the situation one finds with relation to the formation of the spermatia of *Polysiphonia* (71) and similar Florideae. In the latter case the spermatial body is ordinarily regarded as an antheridium which is bodily detached and acts as a unit in fertilization. In *Nemalion*, on the other hand, it is known that the antheridial contents are ejected to form the spermatium which is thus a naked mass of protoplasm. After the spermatium reaches the trichogyne a nuclear division occurs within it (26, 69). In the Ascomycetes more than one method of formation of the spermatia is also found. In addition to the exogenous type of origin which it has been indicated is the most common, various types of endogenous origin of the minute bodies have also been described. In two different species of *Mycosphaerella*, Higgins (46) has reported that two nuclear divisions take place within spermatiferous cells, followed by formation of cleavage furrows which divide the protoplast into four parts. No walls are organized about these, but finally one or more sterigmata push out from the mother cell, and the four young spermatia pass out singly to mature at the apex. Jenkins (50) found the spermatia to be produced endogenously within spermatiferous cells in precisely the same fashion in another species of *Mycosphaerella*. Dodge (35) has described microconidial formation in *Neurospora*; and although he says that the microspores are not formed internally, he states that they appear as if forced out through a collared opening one after another. The situation is not perfectly clear here, but at least it seems evident that the details of the process must differ considerably from exogenous development along lines such as here described in the case of the cherry leaf-spot pathogen. The microconidia of *Botrytis* (22) appear to be formed in much the same fashion that they are in *Neurospora*. In the Laboulbeniales (64) both exogenous and endogenous spermatia are found. The manner in which these bodies are produced is made the basis for separating the families in this order. The exogenous spermatia are cut off

from the ends of more or less specialized branches, and are walled structures. In those forms where the bodies are endogenous in origin these structures are extruded from the antheridial cell as a naked protoplasmic mass. For those who favor the theory of the origin of the Ascomycetes from the Rhodophyceae or would derive the two groups from a common ancestral line, the interesting parallels which exist in the manner of origin of the spermatial elements in the two groups, offer a point which may deserve more careful analysis especially as more forms are studied and more detailed information is accumulated in both plant groups.

Those who favor the red algal ancestry theory of origin of the Ascomycetes and rusts might see new support for their convictions in the recent demonstrations of the functioning of the spermatia to effect fertilization in various members of these two groups of the fungi. Although to date no microconidia have been seen attached to trichogynes in any one of the three ascomycetous forms where the experimental demonstrations in question have been made, preliminary cytological studies have shown prominent trichogynes prolonging coils and extending in such a position as to make copulation with spermatia possible. Further studies will doubtless reveal such copulations actually taking place. In several rusts where the functioning of the spermatia has been proved, attachment of spermatia to receptive hyphae, fusions between these structures, and nuclear migrations have already been reported (6, 30).

Brierley (22) presented good evidence that the microconidia of *Botrytis* will germinate, and Dodge (35) demonstrated that in *Neurospora* these bodies will not only germinate but give rise to ordinary mycelia which produce ascogonial fundamentals and microconidia, and can be mated together to give ascocarps in the familiar fashion.

Those who favor the idea of a monophyletic origin of the fungi undoubtedly find in the increasing number of demonstrations of spermatial germination evidence to support their theory. Some argue that no cells can be true male gametes if they germinate. Dodge (35) has recently discussed the theoretical questions involved. It seems to me that the mere fact that fungus spermatia will germinate need have no implications at all as to their origin, but perhaps rather may be explained on the basis of the plasticity which all the lower plants show and which is notoriously in evidence among the fungi.

The zoologist Dürken (38, p. 31), discussing parthenogenesis in a recent book on experimental embryology, writes: "Es mag dahingestellt bleiben, ob es vielleicht nicht doch Spermien gibt, die in der Lage wären, sich zu teilen, also etwas Analoges zu leisten wie das Ei im Furchungsprozess; jedenfalls ist das aber zur Zeit nicht bekannt und auch eben nicht sehr wahrscheinlich." The facts which have accumulated in the case of the Ascomycetes and the rusts relative to the capacity of spermatia for ger-

mination and vegetative development (2, 4, 6, 22, 35, 47, 52, 61) cannot fail to broaden the ideas of biologists concerning the problem of the ability of male cells to develop alone. Despite the fact that Drayton (37) clearly refers to the microconidia of *Sclerotinia gladioli* as male "gametes," it seems clear that the spermatia in the fungi as well as in the algae may (with the possible exception of some of those of endogenous origin) perhaps best be regarded as reduced antherids. However, they are extremely reduced in size and like true male gametes possess only a very small amount of cytoplasm. Hence as regards the factors limiting free development, the two structures have much in common. Dodge (35, p. 357), commenting on the phenomenon of spermatial germination in *Neurospora*, says: "A microconidium has, barring somatic segregation or mutation, all the potentialities of a monilioid conidium, all the potentialities of the whole mycelium for that matter. If you give a microspore the right cultural conditions there is no fundamental reason except its small size, why it should not germinate." It is well to keep in mind that the entirely different fashions in which the fungus body and the animal embryo begin development might make the reduced amount of cytoplasm a more powerful limiting factor in the one case than in the other. Blackman (16) has pointed out that a complete absence of power of vegetative development is not a necessary feature of male cells. It is well known that potential gametes in some of the brown algae may behave either as sex cells or develop asexually; and in *Ectocarpus*, when they behave as gametes, they may show "relative sexuality" (44). The "zoospores" of *Synchytrium* may also either develop vegetatively or fuse in pairs; in the latter case they too frequently show only relative sexuality.

Dodge (33) has recorded observations on an interesting race of *Caeoma nitens* which produces spermogonia and in which no cell fusions occur at any point in the life cycle. He has referred to this (34) as a "self-propagating androgenetic" race. This is not truly androgenetic, however, in the sense that the thallus results from germinating spermatia.

Dodge (35) was able to show in the case of *Neurospora* not only that the microconidia are able both to act in a sexual capacity and to germinate, but he has proved that ordinary monilioid conidia may effect the fertilization of a sclerotial body (incipient perithecium) as satisfactorily as spermatia will do it. I have had adequate opportunity to convince myself of the correctness of his findings, for in connection with some *Neurospora* studies now in progress I have fertilized sclerotial bodies in hundreds of plate cultures using the "conidization" method. The interesting situation here is certainly a striking example of plasticity. But neither microconidia nor macroconidia are in *Neurospora* necessary to fertilization, for this can be accomplished by direct interaction of compatible mycelia causing diploidization, apparently in the fashion common in the Hymenomycetes

as explained by Buller (24). This situation is to be contrasted with that seen in *Sclerotinia gladioli* where Drayton (37) found that only when spermatia were placed on receptive bodies were apothecia developed.

But neither the fact that microconidia may germinate nor the fact that in at least one case ordinary conidia may bring about fertilization, invalidates the conception that the spermatia are true male elements. Morphologically there is every basis for this view; and one might suppose that the mass of evidence which has grown up in the last few years showing that in many forms in both the rusts and Ascomycetes the spermatia are today functional fertilizing elements, would have added considerable weight in that direction. That in turn the confusion present in the minds of many mycologists and botanists relative to interpretations of phenomena of sex and heterothallism in the fungi, would have been considerably abated, one might also expect. But considerable confusion apparently still exists. And certainly there is no complete agreement among investigators as to how some of the phenomena they observe should be interpreted or in what terms they should be described.

Craigie (28, p. 765), in the paper in which he first reported his epoch-making discovery of the function of the spermatial bodies in the rusts, states: "The pycnia (spermogonia) of the Rust Fungi are not, as many botanists have supposed, male conceptacles producing non-functional spermatia, but are active organs having a non-male function." While Allen has recently begun to substitute "spermatium" for "pycniospore" in her writings, she points out (3, p. 750): "The word 'spermogonium' implies a male structure. It should not be forgotten, however, that when nectar is interchanged between two infections of different sexes, each fertilizes the other." Drayton (37), Ames (7), Gwynne-Vaughan and Barnes (41), Dodge (34, 35), Rice (60), Cayley (25), and others agree at least in unequivocally asserting the male character of the spermatia in the Ascomycetes, or rusts, or both.

Rice points out in a recent paper (60, p. 26) that "in considering the sexual nature of spermatia and microconidia such confusion as Craigie's might be avoided by following C. E. Allen (1) in recognizing the distinction between (a) characters which distinguish gametes, sex organs, or individuals as male or female and (b) characters which regulate syngamy." Interpretation of heterothallism as seen in the higher fungi in terms of compatibilities and incompatibilities which may exist side by side with maleness and femaleness completely obviates the necessity of thinking in the case of certain "heterothallic" Ascomycetes of the existence of two sexes on each of which appear functional organs of reproduction that most workers interpret as male and female, or of thinking, in the case of certain Basidiomycetes, of a species with four sexes, for example. It is encouraging that recently Drayton (37), Ames (7), Gwynne-Vaughan and Williamson

(42, 43), Cayley (25), and others have, in the case of heterothallic species, made interpretations which do not do violence to this mode of thinking.

The word "heterothallic" was coined by Blakeslee (18) in connection with the Mucors. It has since been adopted by the students of the higher fungi, and confusion has resulted. Provided we bar sexual implications and use the word in its etymological sense only, it is still a good convenient term. A heterothallic species is thus simply one in which "thalli of different kinds" exist, the inference being that the interaction of two suitable strains is essential for the initiation of the diploid phase.

From the observations here recorded it is clear that in *Coccomyces* the trichogynes are highly developed structures, showing little or no tendency toward degeneration. Many other examples among the Ascomycetes where a similar situation obtains have already been cited. Let me again point out that in those cases where experiment has shown that the microconidia act as fertilizing agents, also in most of the Laboulbeniales, many of the lichens, etc., trichogynes which end in such a position as to make copulations with spermatial bodies possible, have been found. On the other hand a large number of forms have been reported as showing archicarps which are organized on similar lines but with more or less degenerate trichogynous structures. In some of these forms spermatial bodies are produced; in others they are apparently absent. In some of these a variety of nuclear phenomena have been described which involve the conception of substitute sexuality of one sort or another. Still further we have other cases where an entirely apogamous situation is reported.

The great number of Ascomycetes which show definite antheridial structures will not be discussed at this time. *Ascobolus carbonarius*, Bachmann's *Collema pulposum*, etc., show how such forms may have originated (32). Disciples of Dangeard, holding that the facts of the case best fit in with the idea that the roots of the ascomycete group are to be sought among the lower fungi, must regard these forms with antherids as the more primitive. They consider the microconidia or spermatia to be conidial in nature, as was held by Cornu (27) and Brefeld (21, p. 60-61). If it is ultimately shown that these bodies actually effect fertilization by fusing with trichogynes, then the protagonists of the Dangeard school may be forced to see in these cases a most remarkable example of substitute sexuality!

However all this may be, the situation in *Coccomyces*, as we have seen it, is such as to offer every possibility for fertilization through the agency of the spermatia. The large number of microconidia produced, the definite tendency of the trichogyne to grow toward the acervulus region, and, finally, the exposed position of the trichogyne tip, all combine to suggest a Floridean type of fertilization. The observations reported, especially in the light of the recent work of Drayton, Dodge, and Ames, make highly

desirable further investigation along the lines here outlined, both on *Coccomyces* and other forms showing spermatia and trichogynes.

Morphological studies on the higher Ascomycetes have revealed a wide variety of ascogonial structures; and they have shown that the ascocarp may arise in a variety of ways. The archicarp may be single, and, if multicellular, one or more cells may function as ascogenous cells and give rise to ascogenous hyphae. In other forms several archicarps are associated in the development of a single perithecial or apothecial fructification. In some cases the initiation of the ascocarp begins with the formation of an archicarp or an archicarp associated with an antherid; and enveloping hyphae arise only later. In other cases the development of a stroma marks the beginning of the fructification; and within this, one or more archicarps may appear. *Coccomyces* is of this latter type, although, as we have seen, on rare occasion a coil may develop just outside of the stroma—only to be quickly enveloped. The apothecia of the cherry leaf-spot fungus are also of the compound type. As many as a dozen ascogonia may be involved in the development of a single ascocarp. In some cases we should perhaps regard the fruiting body as doubly compound, since, as described above, it is clear that two or more stromatal patches may fuse together to give rise to a single large apothecial stroma. If spermatial fertilization takes place, the existence of such a large number of ascogonia in a single stroma would seem to make development more certain, on the chances that at least some of them would be fertilized. My studies to date on the development of the ascogenous system would seem to indicate that ascogenous hyphae may arise from at least several cells of a coil. There is some evidence that some of the ascogonia may degenerate.

Since it appears that more than one cell of a coil may give rise to ascogenous hyphae, it becomes a question whether, in the event spermatial fertilization were proved, fertilization of more than one cell could be effected if only one spermatium became attached to the trichogyne. Drayton and Dodge are at present faced with this very problem in even more difficult form, for while in *Coccomyces* all the cells of the coil are, at least in the beginning, uninucleate (Fig. 7 D), in *Sclerotinia* and *Neurospora* they are multinucleate from the start. Drayton (37) has figured the association of the numerous nuclei in the ascogonia of *Sclerotinia gladioli* and assumes one of each pair to be of spermatial origin. In such a situation it is necessary either to assume passage of a nucleus into the trichogyne from each of many microconidia, or postulate divisions of one or a few nuclei of spermatial origin after their entrance into the archicarp.

We have seen that the trichogynes of *Coccomyces hiemalis* show a decided tendency to grow up into the microconidial region above the stromatal patch in which the trichogynes arise. The seeking out of this sper-

matial area, as it were—similar to the tendency which Bachmann (10, 11) noted for the trichogynes of *Collema pulposum* to grow directly toward the spermatial nests deep in the lichen thallus—is strongly suggestive of spermatial fertilization and of homothallism. Other considerations would also appear to indicate that we are here dealing with a homothallic species. For the trichogynes to end in the region where they do—a region which, as has been explained, is occupied by the dense mass of microconidial and macroconidial bodies which are the product of the acervulus—would seem a decided disadvantage should it be necessary for microconidia from some other pustule to become attached to the trichogyne in order for fertilization to be effected, although the situation in certain heterothallic rusts where receptive hyphae have been observed in the spermogonia should lead one to be cautious on this point.

SUMMARY

1. The first indication of the approach of ascocarp initiation in *Coccomyces hiemalis* is to be found late in August or the first of September, when microconidiophores begin to replace macroconidiophores in the acervuli. Evidence is presented to show that the microconidiophores take their origin, at least in most cases, from the macroconidiophores already present. Production of microconidia by normal *Cylindrosporium* spores is reported, but purely microconidial or spermatial fruiting bodies also occur.

2. The microconidia are described as small bacilliform bodies resembling in many features the spermatia of other autonomous Ascomycetes, and those of the lichens, rusts, and red algae with which it is believed they are homologous. In general microconidia of this fungus occur only rarely in culture; but one single-ascospore strain which forms them abundantly in culture was found. All attempts to germinate these bodies failed.

3. The first step in the initiation of the perfect stage involves the development of "stromatal patches" deep in the leaf tissue and in association with the acervuli. Several of these plectenchymatic patches may form in connection with a single acervulus. Each patch is potentially an ascocarp fundament, but fusions between them may occur.

4. Coiled archicarps—as many as a dozen in some cases—appear in each stroma. Each coil is prolonged into a trichogyne which grows up to end usually among the microconidia in the acervulus at the surface of the leaf.

5. In stained free-hand sections the attachment of microconidia to trichogyne-ends was observed. No actual fusions between the spermatial bodies and the trichogynes were seen. The trichogynes, particularly their upper portions, are ephemeral structures. They begin to disintegrate even before leaf-fall.

6. The relations of trichogynes and spermatia in this fungus are such as to indicate homothallism.

7. At leaf-fall a saprophytic mycelium is developed. This usually is not merely a transformation of the old parasitic mycelium; new growth is commonly involved. All the hyphae of the saprophytic mycelium eventually become thick-walled.

8. With the approach of winter a pseudoparenchymatous covering develops about each stroma, and the latter passes into a resting condition.

9. The fungus may be killed or ascocarp initiation and development definitely inhibited by severe freezing, in the case of infected leaves remaining on the tree.

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STARCH DETERMINATION METHODS INVOLVING SOLUBILITY IN ACIDS

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Although previous experiments (2) showed that with certain kinds of plant tissue correct results for starch were obtained by merely digesting the tissue with takadiastase and omitting subsequent acid-hydrolysis of the enzyme-digest, they also indicated that with other kinds of tissue the results were much higher than were obtained by a method based on the removal of the starch from the tissue by hot concentrated calcium chloride solution and on the estimation of the starch in the precipitate formed on the addition of iodine to this extract.

It was desired to make further comparisons of these methods by another method which would be independent of these two, and preferably by one which was based on the removal of starch from the tissue by a solvent other than CaCl_2 . Rask's method (7) was selected because of the favorable results obtained by him (8), Jones (4), Palmer (6), and their collaborators. By this method the starch is extracted from the tissue by cold strong HCl (20.5 to 21.0 g. HCl per 100 cc., i.e., slightly less than one volume of HCl sp. gr. 1.19 to one volume of H_2O); the dissolved starch is precipitated by alcohol, and this precipitate is dried and weighed as starch.

In applying Rask's method to various tissues difficulties were encountered because of incomplete extraction of starch from the tissues, especially from leaves, the starch apparently being enclosed within cells into which the acid did not penetrate readily. Grinding the tissue in a mortar with the acid and making several successive extractions removed the starch from the tissues successfully. But in most cases the estimation of the starch by the weight of the alcohol precipitate could not be depended upon because of the inclusion of non-starch substances. By hydrolyzing this precipitate with acid, or preferably with takadiastase, and by estimating the starch from the copper-reducing power of the sugars formed, the method gave starch values which are believed to represent with fair accuracy the true starch content of the tissues.

SPECIES USED

The tissues used and their preparation for analysis were as follows: Leaves of *Salvia splendens* Ker.; black walnut, *Juglans nigra* L.; tomato, *Lycopersicon esculentum* Mill.; lilac, *Syringa vulgaris* L.; cotton, *Gossypium hirsutum* L.; grape, *Vitis labruscana* Bailey; sunflower, *Helianthus annuus* L.; bean, *Phaseolus vulgaris* L.; peanut, *Arachis hypogaea* L.; soybean,

Glycine max Merr. Seeds of barley, *Hordeum vulgare* L.; malt prepared from barley by allowing the seed to germinate until the roots were about one-half inch long, drying the tissue in a current of air and grinding it in a food grinder; and commercial wheat flour, brand Pillsbury's Best.

The leaves were cut into pieces, were dropped into boiling 95 per cent alcohol, and were extracted thoroughly in a Soxhlet extractor with alcohol. The barley seeds and barley malt, after being ground in a food grinder, were also extracted in the same manner. After the extraction was complete the tissues were dried in air and were ground with a hand mill and with mortar and pestle until all of the material passed through a 60-mesh sieve. The wheat flour was not extracted with alcohol.

TABLE I
RECOVERY OF POTATO STARCH DISSOLVED IN HCl AND IN H₂SO₄

Duration of contact of starch and acid, min.	Temp. °C.	Per cent recovery of potato starch which was dissolved in acid			
		In HCl	In H ₂ SO ₄ (sp. gr. 1.84) (Amount per 100 cc. of starch-acid solution)		
		Rask conc.	32.5 cc.	35 cc.	37.5 cc.
30	20.0	101.0	—	99.0	—
	22.5	98.8	—	100.1	—
	25.0	98.8	—	99.2	99.6
60	20.0	99.2	99.6	100.3	98.9
	22.5	98.0	100.3	99.0	97.5
	25.0	95.1	98.9	97.9	—
90	20.0	96.4	99.8	97.8	97.8
	22.5	93.9	98.6	97.8	97.2
	25.0	93.1	97.2	93.7	93.7

RECOVERY OF POTATO STARCH AFTER DISSOLVING IT IN ACIDS

Since successive extractions of tissue with HCl were found to be necessary in order to obtain a sufficiently complete removal of the starch, tests were made of the limits of time, temperature, and acid concentration within which no serious loss of starch would occur. The percentage recovery of starch dissolved in acid solutions is shown in Table I. Potato starch was first gelatinized by boiling water and the volume was adjusted to give 0.8 per cent starch. Aliquots of 25 cc. each were pipetted into test tubes 30 × 150 mm., and to each were added 25 cc. of double-strength Rask acid (i.e., two times 20.5 to 21.0 g. of HCl per 100 cc. of diluted solution). The acid was added a few cc. at a time with continuous cooling by shaking the tube in cold tap water. Each tube was then removed and shaken under another tap delivering water whose temperature had been adjusted to the temperature at which the starch sample was to be allowed to stand, and finally was placed in a pan of water previously adjusted to this tem-

perature. The count of time began when the addition of the acid to the starch solution was complete.

The temperatures used were 20°, 22.5°, and 25°C., and the duration of contact of the starch with the acid was varied from 0.5 to 1.5 hours. At the end of each period the starch-acid mixture was poured into a beaker containing 150 cc. of cold 95 per cent alcohol, and the tubes were rinsed with 25 cc. of Rask acid, the rinsings together with 75 cc. of 95 per cent alcohol being added to the beaker. After about one hour the contents of the beaker were filtered through a layer of asbestos in a 60-mm. Büchner funnel, and the precipitate was washed with 95 per cent alcohol. The pad of asbestos containing the precipitated starch was placed in a flask by means of pincers and 100 cc. of water were used to complete the transfer from the Büchner funnel to the flask. Ten cc. of HCl sp. gr. 1.125 were then added and the starch was hydrolyzed (1, p. 282). Subsequently the copper-reducing power of an aliquot was determined for each lot (1, p. 379). The control lots were carried through the same procedure except that 25 cc. of H₂O rather than 25 cc. of double-strength Rask acid were added to the starch. To some of the control lots 25 cc. of acid were added to the alcohol after the starch had been poured in. In such control lots the starch precipitated more readily than it did in those containing no acid, but the copper-reducing capacity of the final solutions was not changed. The KMnO₄ titration values for the aliquots of the lots in which the starch had been dissolved in acid were compared with equal aliquots of the control lots in which the starch had not been dissolved in acid, and the ratios of these two values gave the percentage recovery of the starch which was taken originally. These values are shown for Rask's acid in column 3, Table I.

Tests were made also of the solubility of starch in acids other than HCl, and favorable results were obtained with H₂SO₄. Using concentrations of 32.5 to 37.5 cc. of concentrated H₂SO₄ sp. gr. 1.84 per 100 cc. of the final solution obtained by mixing equal volumes of acid and 0.8 per cent potato starch suspensions, it was found that the starch dissolved within about ten minutes to give a water-clear solution from which the starch could be coagulated upon addition to alcohol. Solution of starch occurred less rapidly with 32.5 cc. of H₂SO₄ than with Rask's acid, but with 35.0 cc. of H₂SO₄ the rate was more rapid. Tests of the percentage recovery of starch after dissolution in H₂SO₄ were carried out in the manner described for Rask's acid. The results are shown in columns 4, 5, 6, Table I.

Table I shows that with both HCl and H₂SO₄ recovery of the starch was nearly complete if the temperature did not rise above 22.5°C. and if the period of contact was not longer than one hour. At higher temperatures and for longer periods there was a definite loss of starch. It is also

seen that a fair range in concentration of H_2SO_4 could be used without producing much effect upon the recovery of the starch. Since the solution of the starch occurred rapidly at a concentration of 35 cc. of H_2SO_4 per 100 cc. this strength was adopted for future experiments.

RESULTS BY RASK'S METHOD WITH CERTAIN TISSUES

That approximately correct or entirely incorrect results may be obtained by the Rask method, depending upon the type of tissue which is used, is shown in Table II. The values in column 2 were obtained by the Rask method using the improved procedure recommended by Jones (4), and in columns 3 and 4 are the values obtained by other methods. It is seen that good, or at least fair, agreement was obtained with tomato leaves

TABLE II
UNSUITABILITY OF THE RASK METHOD WITH CERTAIN TISSUES

Plant tissue	Starch as % of the air-dry, alcohol-insoluble material		
	By Rask's method	By other methods	
		CaCl_2	Takadiastase
Salvia, leaves	4.0	11.4	16.8
Black walnut, leaves	0.8	2.1	3.4
Tomato, leaves	13.3	14.3	14.9
Barley malt	52.4	47.8	51.1
Barley seeds	57.6	49.0	54.8
Orange, fruit rind	15.0	0.1	0.6

and with barley malt and barley seeds. However, in the case of salvia leaves the results were much too low and with black walnut leaves only small amounts of starch were dissolved by the HCl . The difficulty with these two tissues was that a single extraction with acid was not sufficient to remove even approximately all of the starch from the tissue.

The results with orange rind are interesting since qualitative tests for starch in this tissue were negative. The Rask method gave high amounts of substances soluble in the acid and coagulable in alcohol. Presumably these substances are mainly pectins and not starch. In view of the common occurrence of pectins in plant tissue it will be realized that a method based on the weighing of the alcohol precipitate will have to be used with caution.

Since the Rask method was developed particularly with reference to the determination of starch in flour, and has been found to give satisfactory results in various laboratories (6), some tests with wheat flour were made in these experiments and the results are shown in Table III. In this test the effect of several successive extractions with HCl was determined. The starch percentages based on the weights of the alcohol

precipitates are shown in column 2, and it is seen that the amount of extractable and precipitable material increased with the number of extractions. After the weights of these precipitates had been obtained the asbestos pads containing the starch precipitates were transferred quantitatively with 100 cc. of H_2O to flasks and the starch was estimated by the acid hydrolysis method (1, p. 282). The values so obtained are listed in column 3 and show that not all of the weight of the alcohol precipitate could be accounted for as starch by this method. In column 4 are shown the values obtained when an equal aliquot of the original extract with Rask acid was precipitated by alcohol and was hydrolyzed not with acid but with takadiastase. Again, the amount of starch found increased with the number of extractions. The small gain between the third and fourth extractions indicated that the limit had been reached.

TABLE III

RESULTS OBTAINED WITH WHEAT FLOUR BY VARYING THE RASK PROCEDURE

Number of extractions with Rask acid	Per cent of starch found when the alcohol precipitate was		
	Weighed on Gooch crucible	Hydrolyzed with acid	Hydrolyzed with takadiastase
1	69.0	67.6	62.4
2	76.6	71.8	69.4
3	77.2	73.6	71.6
4	78.0	73.6	72.0

In order to obtain further comparisons a separate sample of the flour was analyzed by the takadiastase method (2, p. 138), the takadiastase being added to the flour rather than to an extract of the flour. This was done to obviate any uncertainty as to the completeness of the extraction of the starch by the Rask acid. The percentages of starch found by two duplicates by this method were 72.0 and 71.0. Comparing this with the values in Table III it is seen that these starch values were obtained with three to four extractions and with hydrolysis of the alcohol precipitate by takadiastase. Other procedures were either too high because of the inclusion of non-starch substances, or too low because of failure to remove all of the starch from the tissue.

The value 69.0, column 2, line 1, Table III, is that which is obtained by the Jones (4) procedure for the Rask method, and is close to the value 72.0 which we are assuming to represent the correct analysis. The Rask value is low by only about 4.5 per cent. And yet it appears from Table III that this value was reached by two compensating errors, one tending toward low results because of incomplete extraction of starch, and the other tending toward high results because of the inclusion of non-starch substances in the alcohol precipitate.

MODIFICATIONS OF RASK METHOD

The results of experiments in extracting the starch from tissues and in getting a correct measure of the starch in the precipitate which was obtained when the acid-starch solution was poured into alcohol are shown for barley malt, barley seeds, and black walnut leaves in Table IV.

It was found that starch grains dissolved more rapidly in the acids if they had first been gelatinized. Consequently the weighed samples of tissue were added to 25 cc. of H_2O in test tubes (30×150 mm.) which were placed in a boiling-water bath for 20 minutes and then were cooled before the acid was added. By adding 25 cc. of double-strength acid to the water

TABLE IV
MODIFICATIONS OF THE RASK METHOD, AND A COMPARISON WITH OTHER METHODS

Starch method used	Starch as % of the air-dry, alcohol-insoluble material		
	Barley malt	Barley seed	Walnut leaves
I. Starch dissolved from tissue			
A. With HCl , and after precipitation by alcohol,			
a. Precipitate weighed	61.4	67.2	9.65
b. Precipitate hydrolyzed with takadiastase	49.0	54.0	2.10
c. Precipitate hydrolyzed with acid	50.5	53.8	3.12
B. With H_2SO_4 , and after precipitation by alcohol,			
a. Precipitate weighed	62.4	66.4	20.45
b. Precipitate hydrolyzed with takadiastase	50.3	53.3	2.05
c. Precipitate hydrolyzed with acid	50.1	53.6	2.80
C. With $CaCl_2$ and after precipitation with iodine, and removal of iodine			
a. Precipitate hydrolyzed with takadiastase	48.0	51.4	2.12
b. Precipitate hydrolyzed with acid	47.6	50.8	2.53
II. Starch not previously dissolved from tissue, takadiastase added directly to tissue	49.0	54.8	3.39

in which the powdered tissue was suspended the final acid concentration was the same as that required by Rask. Cooling under the tap prevented over-heating in adding the acid. In the subsequent extraction of the tissue by grinding in a mortar with Rask acid the tissue was transferred from test tube to mortar, and, after grinding with sand, back again from mortar to test tube with about 20 to 30 cc. of the acid; by using a fine jet from a wash bottle (in these experiments a 100-cc. test tube) containing the acid, it was not found difficult to make the transfer with this amount of acid. In order to keep the temperature low a large pan of water adjusted to $20^\circ C$. was prepared and the test tubes of tissue and acid were kept in this pan at all times except when the tissue was being ground in the mortar or while the tubes were being centrifuged.

For the experiment shown in Table IV four extractions were made and the acid extracts obtained by extracting and centrifuging were collected in

a volumetric flask which was immersed in running tap water until the extractions were completed (always within one hour from the time of the first contact of the tissue with acid). Then the extract was made up to volume and aliquots were pipetted into three volumes of cold 95 per cent alcohol. Precipitation occurred within a few minutes, but filtration of this suspension was slow and more favorable results were obtained by collecting the alcohol precipitate in a large test tube (30×150 mm.) which was used as a centrifuge tube. When the total alcohol precipitate was collected in the test tube it was washed several times by centrifuging with 95 per cent alcohol in order to remove the acid from the starch.

As is shown in Table IV, one aliquot was used for obtaining the weight of the alcohol precipitate, and in this case a tared Gooch crucible was used for receiving the alcohol precipitate which was then dried to constant weight in an electric oven at 105°C . Other aliquots were obtained for hydrolyzing the precipitate either with acid (1, p. 282) or with takadiastase (2, p. 138), and in these cases the precipitate was collected on an asbestos pad in a Büchner funnel (60 mm.). For the takadiastase lots care was taken to remove all of the alcohol by washing the precipitate with ethyl ether since alcohol interferes with the takadiastase digestion. The asbestos pads containing the alcohol precipitates were transferred quantitatively with H_2O to flasks for the hydrolyses by acid or takadiastase. After the hydrolyses were complete and the solutions were made up to volume, aliquots were taken for the sugar determinations by the Munson and Walker method (1, p. 379) and the dextrose values so obtained were converted into weights of starch by the factor 0.93 (2, p. 139).

In addition to using HCl as proposed by Rask, tests were made with H_2SO_4 . The double-strength acid was prepared by adding 700 cc. of H_2SO_4 sp. gr. 1.84 to 300 cc. of H_2O and, after cooling, adjusting the volume to one liter. This is the concentration used for adding acid to the tissue-water suspension which had been boiled and cooled. There is a greater heating effect with H_2SO_4 than with HCl on adding the acid to the tissue-water suspension, but by adding the acid in small quantities and shaking the test tube under the tap the temperature rise is not serious. The concentration of H_2SO_4 for adding to the tissue in the subsequent extractions after centrifuging the first extraction was 350 cc. of H_2SO_4 sp. gr. 1.84 plus H_2O to make one liter after cooling. Four extractions were made, the decanted extracts were combined, and aliquots were taken in a manner similar to that described for the tests with HCl .

For comparison with these results by extraction of the starch with acids, two other methods were used with the same tissues, the CaCl_2 method, essentially as previously described (2, p. 139) (but with certain modifications of procedure as shown in a subsequent paragraph), and the takadiastase method (2, p. 138). By the CaCl_2 method, the starch is dis-

solved in hot concentrated CaCl_2 solution and is precipitated from the cold diluted CaCl_2 solution by the addition of iodine. After the removal of the iodine the starch is hydrolyzed by takadiastase at pH 4.5 for 44 hours. By the takadiastase method, takadiastase is added to the tissue buffered at pH 4.5 by acetic acid-sodium acetate and after digestion for 44 hours is leaded with neutral lead acetate, is delead with sodium oxalate, and the dextrose formed is estimated and calculated to starch by the factor 0.93.

The results in Table IV show that when use was made of this more efficient method of extracting the starch from the tissue the weight of the alcohol precipitate could not be used as a measure of the starch, but that when the alcohol precipitate was subjected to hydrolysis by either acid or takadiastase, values were obtained which compared favorably with those obtained by the CaCl_2 and the takadiastase. In all cases and particularly with walnut leaves the method of weighing the alcohol precipitate gave entirely erroneous results. But even with the H_2SO_4 -alcohol precipitate of walnut leaves (Lot I B a in Table IV) which gave a calculated starch value of more than 20 per cent, hydrolysis of the precipitate gave starch values of 2.05 and 2.80 per cent, which were comparable to those obtained by other methods.

The results by the CaCl_2 method were in most cases lower than those by other methods but this may be due to its greater specificity for starch. Until further work has been done on this point we must consider the possibility that the CaCl_2 results are correct, and that the others are too high because of the inclusion of non-starch substances.

Good agreement was obtained between hydrolysis of the precipitates by acid and by takadiastase with barley malt and barley seeds. With walnut leaves, however, the takadiastase values were always lower than those by acid hydrolysis. Here, again, it seems possible that the acid hydrolysis values were too high because of the presence of non-starch substances. Further work on this phase of the problem would be desirable.

DETAILS OF STARCH METHODS

The manipulative details of a modified Rask method based on these tests and which was suitable for the estimation of starch in various kinds of tissue are given below, together with a description of the CaCl_2 method including the modifications that have been introduced since the previous description (2, p. 139).

Modified Rask method. Put the weighed tissue into a Pyrex test tube 30 mm. \times 150 mm., add a few drops of water from a wash bottle consisting of a large test tube marked with 10 cc. graduations. Tamp and stir the powder with a glass rod in order to break up lumps; gradually add 20 to 25 cc. of H_2O making note of the amount added. Place the tube in boiling water for 20 minutes and then cool in running tap water. Add an

equal amount of double strength Rask acid (7) if HCl is used, or if H_2SO_4 is used, an equal amount of a solution containing 70 cc. of H_2SO_4 sp. gr. 1.84 per 100 cc. of cooled solution. Add the acid in small quantities shaking the tube in cold water after each addition. After all of the acid is added shake the tube in a pan of water, the temperature of which is 20°C ., and let the tube stand in this pan for about ten minutes. Centrifuge and decant the extract into three volumes of ice-cold 95 per cent ethyl alcohol (need not be exactly 3 volumes). If there are many particles of tissue that do not centrifuge down, decant the liquid through a small pad of glass wool in a small funnel. With the test tube wash bottle which now should contain Rask acid (7) instead of water if HCl is used, or with H_2SO_4 solution containing 35 cc. of H_2SO_4 sp. gr. 1.84 per 100 cc. of cooled solution if H_2SO_4 is used, transfer the residue from the centrifuge tube to a mortar, together with the pad of glass wool containing the particles of tissue, add some sharp sand and grind thoroughly. With a fine jet from the wash bottle containing the acid transfer the tissue back to the centrifuge tube. With practice as much as one gram of tissue can be transferred from tube to mortar and back again with about 25 cc. of acid solution. The tube is again brought to 20°C . in the pan of water and allowed to stand for ten minutes, is centrifuged, and the acid extract is decanted into the same beaker of alcohol together with a volume of alcohol three times that of the decanted liquid. Four extractions are usually sufficient to remove all or nearly all of the starch. After standing 30 to 60 minutes the starch becomes well coagulated and the contents of the beaker are poured through an asbestos mat in a Büchner funnel (60 mm.) and the beaker is scrubbed with 95 per cent alcohol using a rubber-tipped rod. If the filtration of this alcohol precipitate is difficult, as occurs with certain tissues, the precipitate is first collected in a centrifuge tube by repeatedly centrifuging and decanting, and is then washed with 95 per cent alcohol; when this is done filtration is rapid. The precipitate on the asbestos is washed with alcohol to remove the acid and then with ether to remove the alcohol. In transferring the asbestos pad with the precipitate to a flask, first carefully moisten with a *few drops* of H_2O , and then the asbestos may be formed into a roll with a pair of tweezers. With H_2O and a rubber-tipped rod wash out the Büchner funnel and add the washings to the flask containing the asbestos pad. Heat to boiling and cool. Add 10 to 20 cc. of acetate buffer at pH 4.5 and hydrolyze by adding takadiastase solution. The amount of diastase required to convert the starch quantitatively to glucose depends upon the hydrolytic power of the takadiastase preparation and this must be determined by the analyst for the sample of takadiastase used (see 2, p. 135, 136). After digestion with takadiastase the sugar content of the solution is determined. The method which should be used for this sugar

determination and the most suitable factor for converting glucose values to starch is left for the decision of the analyst.

Calcium chloride method. The weighed sample of powder is placed in a mortar, is moistened with water, is ground with sharp sand; using 20 to 30 cc. of water from the test tube wash bottle described in the previous paragraph, it is transferred to a Pyrex test tube 30×150 mm. and is placed in a boiling-water bath for 20 minutes. To this hot liquid 1.5 g. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ for each cc. of water used in transferring the powdered tissue are added and the contents of the tube are brought to boiling over a free flame. The tube is centrifuged while the liquid is hot, and the liquid is decanted into a large beaker. If the liquid contains particles of tissue these may be caught in a pad of glass wool in a funnel. With water from the wash bottle the tissue is transferred from the centrifuge tube to the mortar together with the pad of glass wool with the adhering particles. The residue is again ground in the mortar and returned to the tube for further extraction by adding CaCl_2 and heating in boiling water and over the free flame. About 20 to 30 cc. of water are needed to effect the transfer of the tissue to and from the mortar. The amount needed should be noted from the water levels in the wash bottle so that the required amount of CaCl_2 may be computed. Four extractions made in this way remove the starch from such tissues as have been used in the present experiments. The combined calcium chloride extracts in the beaker are diluted with four volumes of water, and from a pipette a saturated solution of iodine in 10 per cent potassium iodide is added until there is an excess of iodine as shown by a drop of liquid on the end of a stirring-rod and by the color of the foam on top of the liquid. Precipitation usually occurs within an hour but with some tissues it may not occur for several hours. If the amount of the starch-iodide precipitate is large, filtration may be slow. In such cases decant into a centrifuge tube and by repeatedly centrifuging and decanting collect the starch iodide in a centrifuge tube. Wash this precipitate two to three times with 95 per cent alcohol in the tube by centrifuging, and then transfer it to an asbestos pad in a Büchner funnel (60 mm.). Add alcohol and filter to remove a part of the excess of iodine and KI, and then add ethyl ether to remove the alcohol. Carefully moisten the starch with a few drops of H_2O , roll the asbestos pad with tweezers and put it in a flask. Complete the removal of the starch precipitate to the flask by means of water and a rubber-tipped glass rod. Boil the water suspension of starch and asbestos to break up the precipitate and to volatilize the iodine. Cool, add the acetate buffer and takadiastase, and proceed as described in the preceding directions for the modified Rask method. It is unnecessary to effect the complete removal of iodine from the solution before adding the takadiastase. If upon cooling the flask, a light blue color reappears, decolorize with a few drops of dilute sodium thiosulphate before adding the enzyme. This cannot be done, of

course, if the sugar determination method that is to be used subsequently is one based on an iodine-sodium thiosulphate titration. But takadiastase splits starch completely in a solution containing enough iodine to give a distinctly blue color.

RESULTS BY DIFFERENT STARCH-DETERMINATION METHODS

The modified Rask method using both HCl and H₂SO₄ as solvent for the starch was applied to nine different tissues as shown in Table V. For comparison are given the values obtained by the CaCl₂ method, and by the takadiastase method (2, p. 138) upon the same tissues. With tomato leaves approximately the same starch value was found by all four methods.

TABLE V
STARCH CONTENT OF TISSUES BY DIFFERENT METHODS

Leaf tissue	Starch as % of the air-dry alcohol-insoluble material			
	Takadiastase method	CaCl ₂ method	Modified Rask method	
			With HCl	With H ₂ SO ₄
Lilac	6.03	3.86	3.27	4.32
Cotton	4.97	3.92	2.46	3.27
Grape	4.03	1.43	1.60	1.61
Sunflower	4.02	1.72	2.62	2.63
Bean	11.70	6.07	7.04	6.76
Salvia	16.80	11.40	12.40	11.90
Peanut	19.66	18.06	16.34	17.66
Tomato	14.90	14.26	14.36	14.44
Soybean	10.60	9.80	9.80	9.50

With leaves of peanut and soybean, the values were not very different. But with all of the other tissues the values by the takadiastase method were considerably higher. Thus, with lilac, grape, bean, and salvia, the CaCl₂ and the Rask method employing both HCl and H₂SO₄ gave good agreement with each other, and showed starch percentages much below those given by the direct takadiastase method. This is interpreted as an indication of the presence in these tissues of non-starch substances hydrolyzable by takadiastase; and it further emphasizes the possibility that, even without acid hydrolysis following enzyme digestion, takadiastase may give "starch" values which are too high because of the inclusion by this method of non-starch constituents of the tissue.

DISCUSSION

Ling and Salt (5) tested Rask's method with barley and malt, and obtained starch values consistently lower than those obtained by digestion with barley diastase. In some cases tissues that showed starch percentages of 50 to 60 per cent by diastase showed only 10 to 20 per cent starch by

Rask's method. They regard these low values by Rask's method as being due to partial hydrolysis of the starch during the time it is in contact with the acid. The evidence in Table I in the present paper is against this view. It does not seem possible that the conditions of their extractions were such as to cause such an extensive loss of starch by hydrolysis. A better explanation would be that the starch was not properly extracted from the tissue. It would be interesting to know the starch values which could be obtained from these tissues if the Jones (4) procedure for disintegrating the tissue in the presence of Rask acid were followed, or if the tissue were extracted by successive periods of grinding with sand as employed in the present experiments.

Herd and Kent-Jones (3) also found lower values by the Rask method than by barley diastase digestion with several different samples of flour or other cereal products. They were inclined to the view, however, that probably the Rask values were correct, and that the barley diastase hydrolyzed certain non-starch constituents which were not dispersed by the Rask acid. They presented some direct evidence on this point. The present experiments furnish corroboration of the correctness of this explanation by Herd and Kent-Jones.

SUMMARY

Evidence was obtained against the view that the low starch values sometimes found in the analysis of plant tissues by the Rask method are due to the hydrolysis of the starch during the period of contact with HCl. Nearly complete recovery was obtained of potato starch dissolved in HCl, held in solution up to one hour at 20° to 22.5°C., and then coagulated in alcohol. It was found also that H₂SO₄ could be used instead of HCl.

In applying the Rask method to various tissues low results were obtained in some cases but this was due to incomplete extraction of the starch from the tissues by the procedures that have been recommended. By successive extractions practically complete removal of the starch was effected. In such cases estimation of the starch by the weight of the alcohol precipitate was found to be inaccurate because of the inclusion of non-starch substances soluble in the acid and coagulated by alcohol. However, when this alcohol precipitate was subjected to hydrolysis by acid, or preferably by takadiastase, the starch estimation based on the copper-reducing power of the sugars formed furnished values that were comparable to those obtained by other methods.

Starch analyses of 12 different kinds of plant tissue, leaves being used mainly, were made by a modified Rask method, using both HCl and H₂SO₄, and these values were compared with those obtained by the calcium chloride method (in which the starch is extracted from the tissue by hot concentrated CaCl₂), and by the takadiastase method (in which the

enzyme is added directly to the tissue and the resulting increase in copper-reducing power is taken as a measure of the starch without subsequent acid hydrolysis of the enzyme-digest). With certain tissues the same or similar values were obtained by all four methods, but with others the takadiastase values were much higher than those obtained by the HCl, H₂SO₄, and CaCl₂ extraction methods. This furnishes corroboration of the view that with many tissues the takadiastase method gives starch values which are too high because of the hydrolysis by takadiastase of non-starch constituents into substances with copper-reducing power.

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CARBON DIOXIDE STORAGE. VI. LOWERING THE ACIDITY OF FUNGAL HYPHAE BY TREATMENT WITH CARBONIC ACID

NORWOOD C. THORNTON

A number of investigators have shown that the growth of fungi can be retarded by increasing the concentration of carbon dioxide in the atmosphere or culture medium. Brown (2) in his studies on the germination of spores and growth of fungi at various temperatures in the presence of various concentrations of carbon dioxide found a retarding influence of 10 to 20 per cent of carbon dioxide which became more marked as the temperature was lowered. Brooks and coworkers (1), investigating the effect of carbon dioxide upon transit diseases of fruits and vegetables, found considerable reduction in fungous growth whenever the gas was present. Concentrations of carbon dioxide up to 30 per cent were successfully employed to reduce fungous growth. The investigation was carried on not only in standard refrigerator cars during transit, but also in pony refrigerators and on a smaller scale at constant temperature in the laboratory; all tests showed the effectiveness of the gas in reducing fungal invasion of the fruit.

Investigations in the handling of meat and fish in the presence of carbon dioxide has furnished additional evidence of the retarding action of the gas on microorganisms. Killeffer (8) obtained a very marked extension in the storage period of meat and fish both at low and high temperature by storage in carbon dioxide. Callow (4), working with pork and bacon, and Coyne (6) with fish have reported similar results. Moran, Smith, and Tomkins (9) have obtained a decided reduction in the growth of meat-attacking fungi by exposing the tissue to from 10 to 30 per cent of carbon dioxide at 1°C.

Those who have attempted to account for the retarding action of carbon dioxide have done so on the basis that since carbon dioxide is an acid its absorption would bring about an acidification, first of the medium and then of the fungus. This increased hydrogen ion concentration was assumed to be unfavorable for the growth of the hyphae.

However, previous experiments (14) with potatoes, carrots, asparagus, etc., showed that the effect of carbon dioxide upon living tissue, at least with certain concentrations and conditions of storage, is not to increase the acidity of the sap, but to decrease it. The present experiments show that this effect is obtained also with the fungus *Sclerotinia*, the pH of which is changed by carbon dioxide treatment from about 5.6 to 7.2. Furthermore, tests showed that increased alkalinity of the medium is more unfavorable for the growth of the fungus than increased acidity, and the

suggestion is made that the retardation by carbon dioxide is occasioned not by its acidifying action, but rather by its ability to change the reaction of the hyphae in an alkaline direction.

MATERIAL AND METHODS

The fungus *Sclerotinia fructicola* (Wint.) Rehm¹ used in this work was isolated from a peach fruit and has been cultured on potato dextrose agar during the course of this investigation. In preparation for the various treatments, transfers of the culture were made to both liquid potato dextrose medium in 200 cc. Erlenmeyer flasks and to potato dextrose agar in Petri dishes. The cultures were allowed to grow from three to six days at room temperature in order to produce good growth of the fungus above the liquid medium before being exposed to the carbon dioxide. It was found desirable to use for these tests only the hyphae that were not bathed by the medium. Contamination of the cultures was prevented by the usual precautions during transfer and growth. The flasks were closed by cotton plugs which were removed during the carbon dioxide treatment to facilitate the diffusion of carbon dioxide into the flask.

The procedure of treatment of the fungus culture with carbon dioxide was the same as that previously employed for the treatment of plant tissue (13). The carbon dioxide, oxygen, and nitrogen used was obtained from cylinders of the compressed gas. In all tests 20 per cent of oxygen was used and the carbon dioxide and nitrogen varied according to the treatment.

The fungous cultures were held at six different temperatures, 2°, 5°, 10°, 15°, 22°, and 28°C., during the period of exposure to the various concentrations of carbon dioxide. This procedure permitted an observation of the effect of the carbon dioxide on the fungus comparable with that obtained when solid carbon dioxide is used for precooling of fruit for shipment.

The estimation of the pH of the hyphae has been carried out by the use of indicators. In many cases these results were checked by the use of the capillary glass electrode and quinhydrone apparatus. The pH of the culture medium was determined colorimetrically by indicators and electrometrically by the quinhydrone apparatus.

The procedure for determining the pH of the hyphae colorimetrically was to remove a small portion from the surface of the culture and mount it in a drop of distilled water on a microscope slide. A small drop of the desired indicator was then added and the hyphae carefully teased apart to mix the indicator solution and water and to make the mount as thin as possible. A cover slip was then placed over the mount and the slide placed under a

¹ The writer is indebted to Doctor John W. Roberts, Senior Pathologist of the Bureau of Plant Industry, United States Department of Agriculture, Washington, D. C., for identification of this fungus.

microscope. Using a 12 X ocular with objectives giving magnifications of 10 and 45 the change in color of the indicator was observed as it came in contact with the contents of the hyphae. The hyphae to which the indicator was added were compared with hyphae mounted in distilled water without the indicator to obviate a misinterpretation of the color change. The Clark and Lub's series of indicators, as given by Clark (5, p. 94), were used and relied upon for the measurements recorded although other indicators such as neutral red, propyl red, and ethyl red were tested.

Two colorimetric procedures of estimating the pH of the hyphae were employed; the Range Indicator Method advocated by Small (12, p. 47-51), and the observation of the tint of color in the hyphae compared with that produced by buffers at known pH within the range found in the hyphae. The combination of two such procedures afforded a dependable estimate of the pH of the contents of the hyphae.

The pH differences between the treated and control hyphae as observed colorimetrically were checked electrometrically by the capillary glass electrode and quinhydrone electrode. For these tests the mat of hyphae was removed, washed with distilled water, and the excess of water removed with filter paper. In the capillary glass electrode test the mat of hyphae was then folded between the fingers and a drop of liquid squeezed out and drawn up into the capillary. Although such a procedure cannot be considered absolutely reliable, it showed a difference in pH between the control and carbon dioxide-treated hyphae comparable with that obtained in the colorimetric determinations. In the tests using the quinhydrone electrode the mat of hyphae was ground in a mortar with quartz sand, and the juice was extracted by squeezing through cheesecloth. The results of these tests showed differences in pH between the control and the treated hyphae which were in the same direction and of approximately the same magnitude as that obtained with the colorimetric method.

RESULTS

pH CHANGES IN THE HYPHAE

An increase in the carbon dioxide content of the atmosphere around the fungous hyphae brought about a very marked decrease in the hydrogen ion concentration of the living protoplasm. This result is noticeable over a wide range of temperature, but the effectiveness of a small quantity of the gas is greatest at a low temperature. A survey of the data in Table I offers a basis upon which to explain the reason for the greater retarding action of carbon dioxide on fungous growth at the lower temperatures. At 2°C. a treatment of the fungus with 20 per cent of carbon dioxide brought about a decrease in hydrogen ion concentration corresponding to 1.8 pH units, whereas at 15° or at 28°C. a like concentration of the gas brought about a change equal to only 0.6 to 0.8 of a pH unit. Treatment of the

fungus with higher concentrations of carbon dioxide may result in a greater total change in pH, but a large change is obtained by using a low concentration of the gas. This result is of considerable importance where one is attempting to inhibit the growth of a fungus on some fruit such as the peach that is readily injured by high concentrations of carbon dioxide. Concentrations of carbon dioxide up to 80 per cent brought about no measurable increase in pH over that obtained with 60 per cent of the gas.

The decrease in hydrogen ion concentration within the hyphae takes place quite rapidly with the carbon dioxide treatment. The total change in pH as shown in Table I. was found after four hours of treatment, and further treatment to the extent of as much as 120 hours resulted in no additional increase in pH that could be determined colorimetrically.

TABLE I

pH OF THE HYPHAE OF *SCLEROTINIA FRUTICOLA* HELD AT VARIOUS TEMPERATURES DURING EXPOSURE TO VARIOUS CONCENTRATIONS OF CARBON DIOXIDE FOR 24 HOURS

Treatment	Temperature of treatment °C.					
	2	5	10	15	22	28
Control	5.6	5.6	5.6	5.6	5.6	5.6
10% CO ₂	6.4	6.4	6.2	5.8	6.0	6.2
20% CO ₂	7.4	6.8	6.6	6.2	6.4	6.4
40% CO ₂	7.4	7.2	6.8	6.8	6.8	6.8
60% CO ₂	7.6	7.6	7.0	7.2	7.2	7.0

The dead hyphae (determined by testing for plasmolysis and recovery) or hyphae injured during mounting always had a more acid reaction than the living or intact hyphae. The protoplasm of living treated hyphae showed a blue color with brom thymol blue indicator but the dead or injured hyphae took on a deep yellow color. The injured or dead hyphae had a pH of approximately 4.0 as shown by other indicators. The pH of this dead protoplasm varied considerably in the control fungus, but with the treated fungus the results were quite consistent, an observation to be expected because of the acidifying effect of the carbon dioxide which dissolved in the non-living medium.

pH CHANGES IN THE CULTURE MEDIUM

Changes in the pH of the culture medium both before and after treatment with carbon dioxide added some interesting information. At the beginning of the experiment the uninoculated culture medium was determined to be at approximately pH 6.0. After inoculation and with the growth of the fungus for four to six days the pH of the medium was lowered to approximately 4.3. If the culture was exposed to 60 per cent of carbon dioxide at this time the pH of the culture medium was lowered still further until pH 4.1 was obtained. Under the same conditions of temperature and

period of storage of 24 hours 60 per cent of carbon dioxide reduced the pH of the control, a sterile culture medium, from pH 6.0 to pH 5.2. From these results it seems impossible to consider that this acidifying effect of the carbon dioxide could account for the decided retarding action on the growth of fungi obtained with as little as 20 per cent of carbon dioxide.

When the fungous culture grows without carbon dioxide treatment, the pH of the medium, after reaching a low level, begins to increase as the culture becomes older. After a long period of growth the medium is so alkaline that the culture becomes "stale," and the fungus ceases to grow. The formation of ammonia as observed by Brown (3) and the development of car-

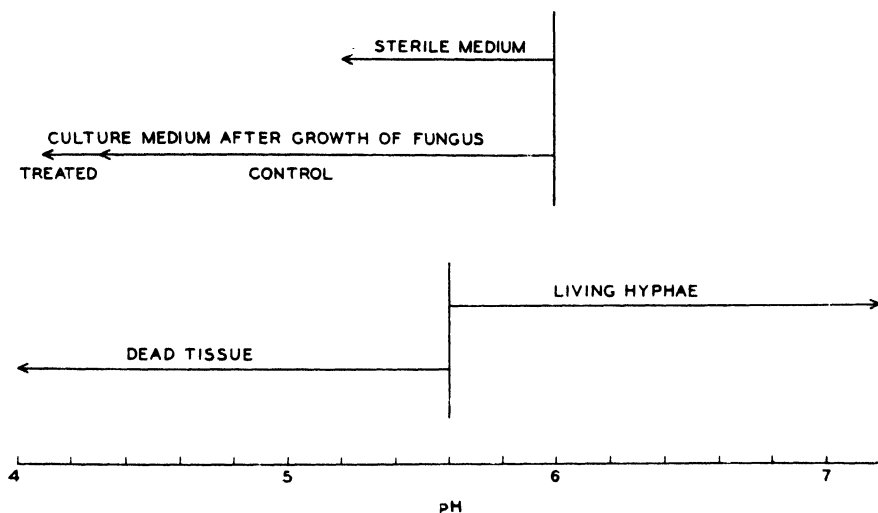


FIGURE 1. The effect of 60 per cent of carbon dioxide on the pH of living and dead hyphae of *Sclerotinia fructicola* and on the sterile and inoculated culture medium during 24 hours of storage at 22°C. The pH of the living hypha and the culture medium at the beginning of the treatment is shown by the vertical lines. The arrow indicating a pH of 4.3 on the line of the inoculated culture medium represents the pH of the medium developed during 6 days' growth of the fungus. The arrow at pH 4.1 represents the additional change brought about by the CO₂ treatment.

bonates as reported by Pratt (10) will bring about the staling of fungous cultures. Many investigators have found that any treatment which results in acidification of the medium aids in continuing the growth of the fungus. The acidifying effect of 5 per cent of carbon dioxide has been used by Brown to promote the growth of stale cultures, but even this method would retard the growth of the fungus if higher concentrations of the gas were used, since the pH of the living protoplasm would be altered.

The effect of carbon dioxide upon the pH of the culture medium and of the living and dead hyphae are shown in Figure 1.

EFFECT OF pH OF MEDIUM UPON GROWTH OF THE HYPHAE

Altering the pH of the culture medium preceding the inoculation with a drop of a spore suspension of the fungus offered further evidence against the view that the retarding action of carbonic acid is due to an acidification of the medium. The fungus grew very well in cultures adjusted to a pH of 4 and 5, more slowly at pH 6, and very little at pH 7. Growth of the fungus at pH 8 was retarded to such an extent that at the end of one week after inoculation only slight development of the hyphae was noticeable. This observation is in accord with the work of Ezekiel (7) who reported that the development of *Sclerotinia* apothecia was favored by an acid medium and reduced or prohibited by an alkaline medium. These results show that the retarding action cannot be due to an acidifying effect of the carbon dioxide upon the culture medium since growth would be favored rather than inhibited by it.

DISCUSSION

In general the effect of carbon dioxide on microorganisms is analogous to the effect of the gas on higher plants; since, according to Rockwell and Highberger (11), its presence is necessary for the growth of the organisms. An increase in concentration above that normally found in the atmosphere may be beneficial even though very high concentrations may become injurious. Although the effect of carbon dioxide on the respiration of fungi is not definitely known, it is to be expected that quite diversified results may be obtained with the various species, and this might aid in establishing the reason for the variation in susceptibility of the different organisms to the retarding effect of carbon dioxide. The physiological changes brought about as an indirect effect of the treatment with carbon dioxide are of a temporary nature and do not appear to be extremely detrimental to the organism since recovery is apparent after removal from the gas. Complete destruction of the culture is not obtained with carbon dioxide alone. Apparently, however, the central or older portion of the fungous culture, or even that portion that cannot become adjusted to the altered atmosphere, may die; not due directly to the carbon dioxide, but to the changes in metabolism brought about by the treatment as shown by the increase in pH of the tissue. The organisms that withstand the change in metabolism recover and continue growth soon after removal from the carbon dioxide to a normal atmosphere.

The acidifying effect of carbon dioxide on the *culture medium*, which has so often been demonstrated and used as an explanation for the retarding action of the gas on fungi, is not unfavorable, but instead is a favorable influence for continued growth. This conclusion is warranted from the observation that *Sclerotinia fructicola* is favored in growth by an acid medium comparable with that produced by the presence of carbonic

acid. Whenever carbon dioxide is present in the culture medium it exerts a further favorable effect in that it retards to some extent the alkalinity developing within the hyphae as a result of the presence of the carbon dioxide. This effect has been shown previously (14) in the study of the effect of the gas on fruits and vegetables. Therefore, it is suggested that it is not the acidifying effect of the carbonic acid upon the culture medium that inhibits the fungous growth, but the alkalinity developed within the hyphae as a result of the effect of carbon dioxide upon the metabolism of the living cells.

SUMMARY

The hydrogen ion concentration of the hyphae of *Sclerotinia fructicola* was determined by the use of the colorimetric method employing Clark and Lub's indicators. The results were also checked with the capillary glass electrode and quinhydrone electrode. The fungus growing on liquid and solid potato dextrose media at six temperatures ranging from 2° to 28°C. was exposed to various concentrations of carbon dioxide with 20 per cent of oxygen.

A decrease in hydrogen ion concentration of the living hyphae from about pH 5.6 to about pH 7.2 was brought about by the treatment with carbon dioxide. The decrease in hydrogen ion concentration was found to be greater at 2°C. than at 15° or 28°C.

Increased alkalinity is more unfavorable for the growth of the fungus than increased acidity, and the suggestion is made that the retardation by carbon dioxide is occasioned not by its acidifying action, but rather by its ability to change the reaction of the hyphae in an alkaline direction.

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CARBON DIOXIDE STORAGE. VII. CHANGES IN FLOWER COLOR AS EVIDENCE OF THE EFFECTIVENESS OF CARBON DIOXIDE IN REDUCING THE ACIDITY OF PLANT TISSUE

NORWOOD C. THORNTON

In a previous experiment (2) in which cut flowers were stored in carbon dioxide atmospheres, changes in the colors of the petals were observed. In later tests (3) it was found that the carbon dioxide treatment of various plant tissues induces a change in pH of the sap in the direction of lowered acidity. It is shown in the present paper that there is a connection between these two facts; that the changes in petal color which are brought about by exposure to high concentrations of carbon dioxide are correlated with the change in pH; that the change in the acidity of the cell sap is made evident by the natural indicator, anthocyanin, present in the tissue.

Since most anthocyanins have a red or purple hue in acid medium and develop a bluish or greenish hue as alkalinity is approached this method provides a ready indication of the change of reaction within the tissue.

The flowers used in this study were as follows: two varieties of roses, *Rosa* (hybrid tea) vars. Briarcliff and Templar; *Verbena phlogiflora* Cham.; pink peony, *Paeonia albiflora* Pallas var. *sinensis* Stend.; and Japanese iris, *Iris laevigata* Fisch. varieties unknown. Some of the roses were obtained from a florist and held at 5°C. during treatment of five days for full development of color. Later the roses and all other flowers used in these tests were cut fresh from the Institute gardens and treated with concentrations of carbon dioxide varying from 30 to 80 per cent by volume in the presence of 20 per cent of oxygen for 18 hours at 22°C. The flowers treated for the shorter period showed the color change in the petals as readily as those exposed for the longer period indicating that the pH change within the cell takes place quite rapidly.

The results given in Table I show the effectiveness of carbon dioxide in bringing about the change in flower color and pH of the petals. In every case there is an increase in the pH of the juice extracted from the petals after treatment as determined by the quinhydrone electrode apparatus. Correlated with this change in pH of the tissue there is a definite change in the color of the petals. The color of the flowers before and after treatment was determined by comparison with the standard colors given by Ridgway (1) and the color nomenclature used in Table I refers to that used by this author. When the young flowers were removed from the carbon dioxide gas to air there was a gradual change in color to the extent that the petals regained approximately their normal color within a few hours.

TABLE I
CHANGE IN THE NATURAL COLOR AND IN THE PH OF FLOWERS AS A RESULT OF TREATMENT
WITH CARBON DIOXIDE

		Treatment of flower with stem in tap water				
		Control	20% CO ₂	30% to 35% CO ₂	50% to 60% CO ₂	80% CO ₂
Rose var. Templar	Color	Pomegranate purple	Some color change at petal edge	A decided change of color at petal edge	Aster purple	Nearly as deep as dahlia purple
	pH	5.24	5.51	5.68	5.61	5.66
Rose var. Briarcliff	Color	Rose pink	Some loss of pink color	Light pinkish lilac	Pale lilac	Hue approximately that of pale Lobelia violet
	pH	5.21	5.66	5.83	5.85	5.88
Verbena	Color	Mathews' purple			Lavender violet	Soft bluish violet
	pH	5.17			5.58	5.63
Peony	Color	Thulite pink			Pale Rhodod- ite pink	
	pH	5.54			6.17	
Iris	Color	Light violet		Violet with Salvia blue in veins	Predominately methyl blue especially in veins and center of petal	
	pH	6.46		6.90	7.20	
Iris	Color	Dahlia purple		Fluorite violet	Royal purple	
	pH	6.47		6.76	7.32	
Iris	Color	Haematox- ylin violet		Bradley's violet with dull violaceous blue in veins	Predominately Roslyn blue with Leitch blue in veins	
	pH	6.34		6.76	6.96	
Iris	Color	Amparo purple		Manganese violet with dull bluish violet in veins	Predominately jay blue with soft bluish violet at petal edge	
	pH	6.41		6.78	7.18	

If, however, older flowers were used or the petals were injured by too long an exposure to the higher concentrations of carbon dioxide wilting occurred without any noticeable change in the color that had developed during the treatment.

Some of the untreated rose petals were ground in a mortar and the juice extracted to test the color reaction upon dropping into buffer mixtures at various pH values. In the acid range, below pH 4.0, the juice from the Briarcliff rose petals is rose pink and as the pH is increased the color changes to a pale lilac, then to nearly colorless, as neutrality is approached. With the extract from the Templar rose petals the color goes from a rose red at pH 4.0 to an aster or dahlia purple with increasing pH. A further test was made on the extracted juices to determine at what pH the least change of color would be observed in the juice as it was added to a buffer mixture. The control juices showed the least color change at pH 4.8 to 5.2 while the juice from the petals of buds treated with 50 per cent of carbon dioxide showed least change between pH 5.5 and 5.8. These results correlate very well with those obtained by the quinhydrone electrode determination shown in Table I.

The change in color of the anthocyanin in flower petals can be used to demonstrate the effectiveness of carbon dioxide in decreasing the hydrogen ion concentration of the tissue.

SUMMARY

The effectiveness of carbon dioxide in decreasing the hydrogen ion concentration of living tissue was demonstrated by the change in the color of the natural indicator, anthocyanin, present in the cells of flower petals of rose, verbena, peony, and iris. This change in hydrogen ion concentration of the petals, as much as 0.8 of a pH unit, during the treatment was shown also by pH determinations made upon the juice extracted from the petals, using the quinhydrone electrode apparatus.

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THE SERUM REACTIONS AS AN AID IN THE STUDY OF FILTERABLE VIRUSES OF PLANTS¹

HELEN PURDY BEALE

One of the most important systems of classification in biology is based upon morphology, but with organisms of less complex structure than the higher plants, for example bacteria, although morphologic characters may serve as a gross means of systematic arrangement, other criteria such as biochemical reactions, pathogenicity tests, and serologic behavior are employed ultimately for a distinction between closely related forms. In some instances of classification, the morphologic basis is not applicable, as illustrated by the well known physiologic strains of rust fungi, indistinguishable on the basis of structural characters but exhibiting marked differences in pathogenicity.

In classifying the filterable viruses of plants, agents either of unrecognizable form or ultramicroscopic in size, the morphologic basis is obviously of no value. Investigators of this group of infectious principles must resort to other means of identification (8, 17, 18, 28, 31). As aids in classification, bacteriologists attach considerable importance to cultural characteristics, biochemical reactions, and metabolic requirements as determined by artificial cultivation. The failure of filterable viruses to multiply in ordinary culture media in the absence of living cells renders this method of procedure likewise impossible. Largely because of these handicaps in obtaining information directly pertaining to the infectious agent in the field of filterable viruses, extensive investigations have been directed into the variability of symptom expression, exhibited by a given virus over a wide host range. A common ground for the identification of both bacteria and filterable viruses is their resistance to various chemical and physical agents. Valuable information concerning many of the infectious principles of virus diseases of plants has been obtained by studies of insect vectors, pathologic histology of the hosts, and cell inclusions so characteristic of certain virus diseases.

Although data useful in the differentiation of the filterable viruses are rapidly accumulating through the channels mentioned above, all possible additional means of acquiring further information should be considered. Sufficient positive evidence, which will be reviewed in detail, has already been acquired to recommend the method of serum diagnosis.

The serologic technique has been used to advantage in the identifica-

¹ Joint contribution of the Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, and the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York, New York.

tion of bacteria. To quote Topley and Wilson (32, p. 221, 222): "In certain groups of bacteria, the serological method is found to be much the quickest and most satisfactory way of distinguishing between the different members, and it is therefore extensively used for rapid identification. In other groups the agglutination method is not of much help, either because there are numerous varieties within the species, or because the organisms are autoagglutinable, or for some other reason. Apart from affording a rapid means of identification of certain bacteria, the serological method is as a rule the most delicate method available for bringing out the finer distinctions between closely allied organisms. In this respect it is more valuable even than the biochemical tests. It is often the only method available for differentiating between the sub-species or varieties of a given species of organism."

SERUM REACTIONS AS QUALITATIVE TESTS

In the agglutination of bacteria by a specific antiserum, we have visible evidence of the active participation of the bacterium in the reaction, whereas, in the precipitation of virus extracts by homologous antiserum, there is no direct proof that virus itself plays an active part. So long as the filterable viruses remain unknown entities, it is possible that the precise rôle occupied by them in the serum reactions will remain open to debate. However, the nature of the reaction may be such as to offer real assistance in the identification of these infectious agents and is therefore worthy of most careful consideration.

A number of different investigators have shown that certain of the virus extracts of plants are antigenic in nature, i.e., capable of inciting the formation of antibody when introduced parenterally into animal tissue. The resultant *antibody*, appearing in the blood serum or body fluids of the hyperimmunized animal, reacts specifically with the *antigen* in some observable way. In every case reviewed here, the rabbit has been employed for the production of antiserum. Three types of immunologic reactions have been considered, namely, alexin-fixation, precipitation, and neutralization of pathogenic properties. In precipitation, the antibody is referred to as *precipitin*, and it is with the precipitin reaction that this paper is largely concerned.

In 1927 Dvorak (7) produced antisera separately to extracts of potato plants, that were either healthy or affected with mosaic disease. Precipitin reactions showed that the two extracts had antigenic substances in common, but that both antisera exhibited a higher titer for the homologous than for the heterologous antigen. For example, antiserum to potato mosaic juice showed a higher titer for extract of mosaic-diseased potato than for the juice of healthy potato. The explanation of Dvorak for these results was that the virus of mosaic disease had in some way altered the precipitability of the globulins present in the healthy potato.

In 1928 (26), and 1929 (27), Purdy produced antisera separately to extracts of healthy tobacco and tobacco affected with a virus of mosaic disease [classified by Johnson (17) as Tobacco virus 1]. Antigenic substances were found, common to both extracts, but it was shown for the first time by alexin-fixation tests and the technique of precipitin-absorption, that there was some antigenic substance in tobacco virus extract, specific for virus extract and not present in healthy tobacco juice. The same author demonstrated further that extracts of tomato, pepper, and petunia, in which Tobacco virus 1 had been multiplied, also reacted specifically with precipitin to tobacco mosaic virus extract. The neutralizing action of antiserum to virus extract on the infectious agent of tobacco mosaic disease was also studied. Normal rabbit serum was found to possess practically no inactivating action, antiserum to healthy tobacco showed some power of neutralization, but antiserum to tobacco virus extract, under the conditions of the experiments, produced a marked inactivation of the active pathogenic principle of mosaic, as determined in every case by the subsequent inoculation of healthy tobacco plants. From the results of these experiments, the suggestion was made that the specific substance in virus extract not present in healthy tobacco juice might be either a tobacco protein, altered by the action of the virus, or a foreign virus protein.

In 1930, Matsumoto (19) produced antiserum to tobacco mosaic virus and likewise demonstrated precipitins to the homologous antigen. He found no demonstrable precipitin to healthy tobacco juice. His experiments were chiefly concerned with an investigation of the neutralization of virus by antiserum. Matsumoto was able to show that antiserum to mosaic juice removed virus from the supernatant fluid in an antiserum-virus mixture in which flocculation had occurred. He did not regard this reaction as proof of specific action of the virus, but suggested that concomitant antigens might play a part. The investigations of Matsumoto further determined the effect upon the inactivation of virus produced by such factors as length of incubation period, concentration of antiserum, and temperature at which the antiserum-virus mixtures were held. The failure to obtain inactivation of the virus after such long periods of contact between antiserum and virus as one hour at 37°C. followed by 23 hours in the cold room (19, p. 297) is contrary to our findings. The discrepancy would seem to be due primarily to a difference in the treatment of the inoculum. Antiserum-virus mixtures were incubated in this laboratory for one hour at 37°C. or several hours (4 to 6) at room temperature and, following plant inoculation, an average of 88 per cent of the 89 plants remained healthy (27, p. 931). The entire tube contents were inoculated after thoroughly shaking the precipitate that had settled in the bottom, whereas Matsumoto centrifugalized the mixture and resuspended the precipitate in water before inoculation. Investigators of other viruses have

shown that dilution of antiserum-virus mixtures rendered inactivated virus again active provided the reaction had not been carried too far (32, p. 584). Suspension in water of the precipitate, which has been shown to contain tobacco virus, would probably have a similar effect. However, the method employed by Matsumoto provides a better means of studying the effect upon virus of varying periods of contact with antiserum.

In 1930, Matsumoto and Somazawa (21) proved the identity of the specific antigenic substance in virus extracts of tobacco and tomato, by the production of specific antisera to each extract and the complete absorption of precipitin from both antisera with either antigen. Studies were made on the effect of formalinization, trypsinization, and in a later paper (22) heat-inactivation on the antigenic property and infectivity of virus extracts of tobacco and tomato. The results in brief showed that formalinization tended to lessen both antigenicity and infectivity of virus extracts, but to a lesser degree in the case of the former. The reverse was true with trypsinization, for when virus extracts were subjected to the action of 5 to 10 per cent trypsin, the infectivity of the virus proved more resistant than the antigenicity of the virus extract. The ability of trypsinized virus extracts to react with precipitin in an antiserum to untreated mosaic juice was not appreciably altered by the action of the enzyme. In respect to heat-inactivation, the virus extracts behaved differently, depending upon the treatment. With a temperature of 90°C. for ten minutes, no appreciable amount of antibody was demonstrable in antiserum to the treated mosaic juice, by either complement-fixation (alexin-fixation) or precipitin tests. Such heat-inactivated extracts likewise failed to react with antiserum to untreated virus extract. Positive reactions occurred in both instances, however, as the temperature was lowered or the interval of treatment appreciably shortened. Reference to the tables in a recent article by Price (25) affords an excellent means of determining the degree of inactivation likely to occur with various extracts of Tobacco virus 1, subjected to different temperatures for unequal periods of time. Matsumoto and Somazawa (22) further demonstrated that when formalinized, trypsinized, and heat-inactivated virus extracts were employed as antigens, practically no virucidal antibodies were formed in the antiserum. From the results of these experiments, the investigators have concluded that it seems likely that the virucidal antibodies have little or no relation to the precipitin or complement-fixing antibodies.

As the authors themselves state, the above described experiments were undertaken to determine whether the antibodies were formed against the infective principle of virus *per se*, or whether the flocculation phenomenon in the precipitin reaction was due to the presence of concomitant antigens. Also, more information was desired relative to the nature of the infectious principle as judged from the serologic behavior. By way of reiteration, the

results obtained with formalinized virus extract showed that mosaic juice which had apparently lost its infectivity, still retained some of its antigenicity. Trypsinization, however, lessened the antigenicity of the virus extract more than its infectivity, while scarcely altering the ability of a trypsin-treated extract to precipitate specific antiserum. The authors have suggested by analogy from similar reactions in other fields, that the retention of the precipitating power with an accompanying decrease in antigenic activity might be attributable to "a certain specific substance (carbohydrate?) which remained almost unchanged in the course of trypsinization" (22, p. 24).

In 1931, Beale (née Purdy) (1) performed further investigations as to the specificity of the precipitin reaction in tobacco mosaic disease. Leaf extracts of Sudan grass, *Hippeastrum*, lily, and abutilon, each affected with its respective mosaic disease, failed to give a positive reaction with antiserum to Tobacco virus 1, indicating that the specific precipitating substance present in tobacco mosaic juice was at least not a common constituent of all virus extracts. It was also shown that extracts of cucumber mosaic virus and ring-spot virus, generally regarded as distinct from Tobacco virus 1, did not contain the specific precipitating substance, although the three viruses were multiplied separately in a common host, *Nicotiana tabacum* L. var. Turkish. Still other hosts, *Nicotiana glutinosa* L., *N. rustica* L., and *Martynia louisiana* Mill., were added to those already mentioned in which the tobacco virus has been multiplied and the specific substance demonstrated. The author has since tested a number of virus extracts for the presence of the substance reactive with precipitin to tobacco mosaic juice. The results of these tests, previously unpublished, are reported in Table I. *Asclepias curassavica* L., *Datura stramonium* L., and *Solanum melongena* L. var. Black Beauty all affected with mosaic diseases believed to be distinct from tobacco mosaic virus yielded extracts giving a negative precipitin reaction with antiserum to Tobacco virus 1 while extracts of *Solanum sisymbirifolium* Lam., *Physalis peruviana* L., *Capsicum minimum* Blanco, *C. frutescens* L. var. Pimiento, and *Solanum melongena* L. var. Hangchow Long all reacted positively. The extracts of the last five plants produced local lesions when inoculated on *Nicotiana glutinosa*, symptoms characteristic of Tobacco virus 1 (15). *Solanum melongena* var. Hangchow Long exhibited no macroscopic symptoms of mosaic disease. The material for these extracts was kindly provided by Dr. F. O. Holmes, from plants he had grown out-of-doors during the summer of 1931, with the exception of *Solanum melongena* var. Hangchow Long, affected with tobacco mosaic, which was grown indoors. The author of the present article has also tried the precipitin reaction (see Table I) with extracts of aucuba mosaic virus and the attenuated or masked strain of tobacco virus recently described by Holmes (16). Extracts of aucuba

TABLE I

PRECIPITIN TESTS WITH ANTISERUM TO TOBACCO VIRUS 1 AND EXTRACTS OF PLANTS AFFECTED WITH VARIOUS VIRUSES; ANTISERUM DILUTED 1:1

Antigen		Precipitate*
Host	Disease	
<i>Asclepias curassavica</i>	Mosaic	o
<i>Datura stramonium</i>	Mosaic	o
<i>Solanum melongena</i> var. Black Beauty	Mosaic	o
<i>Solanum melongena</i> var. Hangchow Long	Tobacco mosaic**	+++
<i>Solanum sisymbirifolium</i>	Tobacco mosaic**	++
<i>Physalis peruviana</i>	Tobacco mosaic**	++
<i>Capsicum minimum</i>	Tobacco mosaic**	+++
<i>Capsicum frutescens</i> var. Pimiento	Tobacco mosaic**	++++
<i>Nicotiana tabacum</i> var. Turkish	Aucuba mosaic**	++++
<i>Nicotiana tabacum</i> var. Turkish	Attenuated tobacco mosaic**	++++

* o = no precipitate; ± = very slight precipitate; + = slight precipitate; ++ = moderate precipitate; +++ = heavy precipitate; ++++ = very heavy precipitate.

** Virus extracts of plants induced local lesions, typical of Tobacco virus 1, when leaves of *Nicotiana glutinosa* were inoculated.

mosaic virus showing pronounced mosaic symptoms on Turkish tobacco and the attenuated virus, with masked symptoms on the same host, both heavily precipitated antiserum to Tobacco virus 1.

The ability of the three extracts to give positive precipitin reactions with the antisera to one of them is indicative of the presence of common precipitating substances but no evidence of the identity of the three antigens. Cross titration with absorbed antisera would be essential in determining the identity of the precipitating fraction of the three virus extracts. From the foregoing results the writer has concluded that the specificity shown by the precipitin reaction favors the interpretation that the specific antigenic substance is foreign material, possibly virus itself, rather than altered host protein.

The value of the serologic test is at once evident as a means for the rapid identification of Tobacco virus 1 in "carriers" as well as in unrecognized or new hosts. In studying other viruses where the plants might become contaminated with the tobacco virus, this test would likewise have practical application.

In 1932, Matsumoto and Somazawa (23) made a study of the distribution of the antigenic substance present in tobacco plants affected with mosaic disease. Antigenic material was recorded in leaves, stems, roots, and flower buds. Active virus, as well as antigenic substance, was demonstrable in parts of plants exhibiting no macroscopic symptoms. The antigenic material and virus occurred together in the samples tested, and the authors suggested that the serum-precipitin reaction might therefore be applied to measure virus concentration. The authors also state in con-

clusion that from these data it is inferred that the antigenic reaction is actually due to the infective agent, and thereby may favor the interpretation of Purdy Beale that "the antigenic substance of tobacco mosaic is possibly virus or infective principle itself and not altered host protein." (23, p. 167).

In the same year Matsumoto (20) recommended the serologic method as a means of early diagnosis of tobacco mosaic, since the specific antigenic material had previously been demonstrated in tobacco plants before the development of macroscopic symptoms.

In 1933, Matsumoto and Somazawa (24) reported the results from further studies on the distribution of antigenic material in tobacco plants affected with mosaic disease. By ringing the stems of tobacco plants, and inoculating either above or below the ringed portion, they were able to demonstrate the ability of the antigenic material, which they regarded as probably virus itself, to move freely upward and downward through the lesion. However, when the cortex and central portion of the stem were both removed, leaving only the xylem ring to connect the upper and lower portions of the plant, antigen and virus were detected only in that part of the plant inoculated. An examination of the host tissues from affected plants growing under normal conditions, i.e., without ringed stems, showed antigen in cortex, xylem, and central portion of the stem. The authors infer from these observations that under normal circumstances, virus may pass through the pits in the walls of the xylem vessels and across the medullary rays, but that this movement may be inhibited when the central portion of the stem is removed.

In 1933, Silberschmidt (30), in a treatise on the subject of antibodies in plants, described his own attempts to produce passive immunity in tobacco plants by treatment with rabbit antiserum to tobacco mosaic juice. He was unable to confer immunity of this type on the plant. In other tests the antiserum and virus were rubbed separately on the leaves of the plant and a certain diminution in the number of resultant local lesions was noted. However, in the absence of more complete evidence Silberschmidt felt that the action of the antiserum should be regarded as non-specific. He also demonstrated that virus was precipitated to a greater extent by the action of the specific antiserum than by either normal rabbit serum or antiserum to healthy tobacco juice. He further showed that the amount of precipitate formed was determined by such factors as titer of antiserum, length of contact between antiserum and virus, and relative proportions of the reagents. In antiserum-virus mixtures as a rule, virus was found only in the precipitate but when an excess of virus extract was added to the antiserum the supernatant fluid was also infectious. The infectivity of virus extract was completely or partially destroyed by the action of specific antiserum and much less markedly affected by normal

serum or antiserum to healthy tobacco juice. His results indicated, as he stated, that specific antiserum to virus extract not only possessed flocculating ability but also virucidal properties. The latter quality was reported enhanced if special care of the rabbits were taken during hyperimmunization. Relative to the nature of the virus, judged from the standpoint of serologic behavior, Silberschmidt concluded the discussion of his results with the statement: "... so scheinen mir die serologischen Erfahrungen über die Wirkungsweise des Antivirusserums gegen Virus *in vitro* trotz mancher Widersprüche zwangloser mit der Annahme einer organisierten als der einer substantiellen Natur des infizierenden Agens der Viruskrankheiten vereinbar zu sein." (30, p. 175).

An adequate explanation for the mechanism of the inactivation of viruses in general by their respective antisera has long been sought. The problem when studied *in vitro* is further complicated in the case of tobacco mosaic virus by the presence of precipitin in the specific antiserum. It is, therefore, difficult to prove whether the virus is precipitated in an antiserum-virus mixture by direct interaction between virus and specific antibody, by precipitation of protein carriers of the virus, or by the flocculation of concomitant antigens followed by an adsorption of virus to the floccules. It is, likewise, not easy to determine whether the diminution in virus infectivity is attributable to virucidal antibodies entirely distinct from precipitins. It has been shown that tobacco virus is inactivated by appropriate treatment with various adsorbents and is precipitated from virus extract upon the addition of suitable salts. When an excess of talc, for example, is added to virus extract and allowed to stand overnight, upon filtration the following morning the filtrate, when tested on plants, is found to be non-infectious. Resuspension of the talc in a volume of water equal to that of the original extract is likely to yield an inoculum of greatly reduced infectivity. Even with antisera to virus extract having low precipitin titers, such as those produced by Silberschmidt, it seems probable that the precipitate formed is greater in amount than that formed in mixtures of antiserum to healthy tobacco juice and virus. Likewise, the latter would yield more precipitate than mixtures of normal serum and virus. It is in antiserum-virus mixtures of precisely this order that the virucidal activity decreases. Assuming for the moment that virus is precipitated from serum-virus mixtures by adsorption to the floccules, then the greater the amount of precipitate, the larger the adsorbing surface is likely to be. Further research into the general question of the mechanism of adsorption will undoubtedly benefit the problem of inactivation of the virus by specific antiserum.

In three communications by Gratia (10, 11, 12) published in 1933, the production of separate antisera to a potato mosaic and potato leaf-roll extract, and extracts of two tobacco mosaic viruses were reported. The

subsequent cross titration of antigens and antisera brought out the fact that precipitin and neutralizing antibody specific for tobacco virus extract were demonstrable in the antisera to both tobacco virus extracts but not demonstrable in either of the antisera to the potato viruses. Conversely, precipitin and neutralizing antibody specific for potato mosaic virus or potato leaf-roll were present in antisera to the corresponding potato virus extracts, but absent from antiserum to tobacco mosaic virus. No positive precipitin reaction was obtained between any of the antisera and a virus extract of beet, affected with a mosaic disease. From these results the suggestion was made that the serum reactions would be useful in the identification of filterable viruses of plants as in the classification of bacteria. Gratia further stated he believed from a consideration of the serologic behavior of the plant viruses as well as from that of bacteriophage with which he has also worked, that the specific antigenic substance in virus extract and in phage extract is exogenous in origin. Also specific precipitin reactions were obtained with extracts of different hosts in which the same virus had been multiplied. He concluded that plant viruses, like bacteriophages, could multiply in more than one host but still not lose their antigenic specificity.

In one of two notes published in 1934, Gratia has drawn an analogy between bacteriophages and filterable viruses of plants (13). He maintains that the multiplication of the infectious agents in both cases is dependent upon the rate of growth of the host, and that "masked" carriers have been found for both bacteriophages and plant viruses. Also, upon exposure to both disease entities an acceleration of growth occurs in the host. As arguments against the theory that the infectious agents are of endogenous origin, he cites the antigenic specificity of the extracts of bacteriophage and plant viruses, and also the fact that, in the case of certain of the plant viruses, a period of incubation in the body of an insect is a necessary prerequisite to successful transmission of the disease. Gratia expresses the belief that viruses penetrate cells during mitosis, which accounts for the intimate association between virus and the young cells of the host. In the second article (14), Gratia describes a method of precipitin-absorption, using crushed pulp of healthy tobacco instead of the clear plant extracts employed by previous investigators to eliminate that fraction of the precipitate due to non-specific substances in the virus extracts. He also states that formalinized tobacco mosaic virus retains its specific antigenicity.

In 1934, Birkeland (4) described his serologic studies of plant viruses, whereby he was able to show that a purified preparation of Tobacco virus 1 retained its antigenic specificity, but if the virus extract lost its infectivity by passage through a Seitz filter, the non-infectious filtrate was incapable of reacting with antiserum to virus extract. The purified antigens were reported free of non-specific substances, present in the crude ex-

tracts and common to both healthy and mosaic-diseased plants. Further experimentation with other viruses resulted in the production of antisera to spot-necrosis (Tobacco virus 4 Johnson), cucumber mosaic, ring-spot (Tobacco virus 5 Johnson), and attenuated forms of spot-necrosis and Tobacco virus 1. Subsequent precipitin tests with the antisera and homologous and heterologous antigens indicated a high specificity for the reacting substance in Tobacco virus 1 extract, since no positive precipitin reaction was obtained upon cross titration with any of the other viruses or antisera. The attenuated forms of Tobacco virus 1 and spot-necrosis showed no serologic distinction from the non-attenuated forms. A relationship between spot-necrosis and ring-spot was shown by the positive reactions obtained between antisera to spot-necrosis, both attenuated and non-attenuated, and ring-spot antigen. In conclusion, Birkeland (4, p. 436) states that the close association between antigenicity and infectivity and the specific nature of the antigenic fractions of different viruses suggests that "this specific antigenic factor is either the virus itself or a virus-plant-protein complex in which the virus plays the rôle of a haptene." He believes that the precipitin test should prove a valuable means of classification of plant viruses.

While the evidence at hand, reviewed in this article, is no direct proof that the specific antigenic substance in virus extract is virus itself, nevertheless the antigenic substance present in virus extract is sufficiently specific in immunologic behavior to render its presence valuable as a means of identification of certain viruses. As the serologic method of investigation is continued, it seems entirely probable that technical difficulties will be encountered, whereby it may not be possible to induce observable antibody against certain of the viruses. For instance, in earlier preliminary attempts, the present author has not been successful in obtaining antisera of workable titer for extracts of cucumber mosaic, ring-spot virus, aster yellows, and peach yellows, following the simple procedure used for the production of antiserum to Tobacco virus 1. This should not prove altogether discouraging, in the light of more recent achievements in immunology dealing with methods of increasing antigenicity by the use of adsorbents and by coupling with proteins. Also, the method retains its value where specific antibody is readily demonstrable.

While the serum reactions will undoubtedly have their limitations in the identification of plant viruses, just as in the case of bacteria, the limited evidence to date would seem to justify the recommendation of this technique as an additional aid in the classification of filterable viruses of plants.

SERUM-PRECIPTIN REACTION AS A QUANTITATIVE TEST

In addition to the contribution of the serologic technique as a qualitative test, it has been proposed by several workers as a quantitative method

for determining the concentration of Tobacco virus 1. A preliminary report of the method now to be described in detail has already been published (2).

Heretofore, methods of measuring virus content were based upon pathogenicity tests, in other words the virus concentration of tobacco extracts was determined by the ability of the extracts to retain their infectivity upon dilution. The need of more precise methods of determining virus concentration was generally recognized.

The observation of Holmes (15) that tobacco virus produces local lesions in certain of its hosts is a contribution of great practical importance. Briefly, he has shown that if a piece of cheesecloth moistened thoroughly with virus extract is rubbed over the upper surface of *Nicotiana glutinosa* L., localized primary necrotic lesions will develop in a few days at each place of successful entry of virus into the host tissue. By counting the lesions, he is able to demonstrate a relationship between the number of resultant lesions and the virus concentration of the inoculum. Holmes' article (15), and recent papers by Samuel and Bald (29) and Caldwell (5) suggesting certain modifications in Holmes' original method, have contributed a great deal to improved methods of measuring virus concentration. All of the published methods to date allow a certain orderly arrangement of two or more virus extracts, with varying degrees of precision and within certain limitations. No method has proved entirely adequate in answering the questions "What concentration of virus is present in a given sample?" and "How small a percentage difference in virus concentration is demonstrable?" The author of the present article has attempted an answer to the first question by resorting to the serologic technique. The success of the serologic method depends upon the possible quantitative relation between the amount of precipitating substance in a given dilution of virus extract when mixed with the homologous antiserum, and the virus content of the same extract as determined by its ability to induce local lesions when rubbed on *N. glutinosa*. In an effort to provide a partially satisfactory answer to the second question, it was necessary to resort to the inoculation of a number of extracts of known concentration and observe the differences in concentration that were demonstrable by the method employed.

The serologic method used in determining the antigenic content, or in this particular reaction the precipitating fraction of the virus extracts, will be described first, and later the procedure used in estimating their virus concentrations by the local lesion method will be presented.

DETERMINATION OF PRECIPITATING FRACTION OF VIRUS EXTRACTS

Preparation of antigen. Johnson's Tobacco virus 1, multiplied in Turkish tobacco, was used as the source of antigen. In some cases leaf extracts were prepared by grinding the green tissue to a pulp in a mortar with

saline (0.85 per cent) solution and filtering the extract through paper, the final clear filtrate being obtained by passage through a double filter, consisting of Whatman's Nos. 5 and 42 papers. In other cases the green tissue was minced and added to the saline and allowed to freeze and thaw alternately several times before final filtration through the double filter. The latter method of extraction seemed to produce clearer extracts with greater facility. The proportion of saline added to 10 grams (green weight) of tissue varied from 50 to 100 cc. and was recorded in every case. Phenol was added to the crystal clear filtrates to a concentration of 0.5 per cent. The preparations were corked and stored in the ice chest.

TABLE II

PRECIPITIN TESTS FOR PRELIMINARY ESTIMATION OF ANTIGENIC CONTENT OF VIRUS EXTRACTS

Tube No.	Cc. antiserum 1:50	0.3 cc. antigen dilution	Precipitate*
1	0.3	Undil.	++++
2	0.3	1:4	+++
3	0.3	1:8	++
4	0.3	1:16	+
5	0.3	1:32	+
6	0.3	1:64	o
7	0.3	1:128	o
8	0.3	1:256	o
9	0.3	Saline	o
10	Saline	Undil.	o

* See Table I, footnote *.

Titration of antigen. Three-tenths of a cc. of increasing dilutions of virus extract, as indicated in Table II, were mixed with 0.3 of a cc. of a 1:50 saline dilution of antiserum to obtain an approximate estimation of the precipitating fraction of the virus extract. After incubation in a water bath at 37°C. for one hour or at 56°C. for two hours, the tubes were thoroughly shaken and placed in the ice chest overnight. The following morning the tubes were examined for the presence or absence of precipitate. The experiments were then repeated using decreasing amounts of the highest dilution of antigen at which a positive reaction was obtained in the preliminary tests (Table III). If at any time during the experiments the stored antigens became cloudy and precipitated out they were refiltered before use, since the results obtained by the use of cloudy antigens were unreliable. The refiltered preparation was tested again for its precipitating fraction since the precipitin test is so delicate that loss of antigen has been detected following the filtration of preparations containing heavy sediments, whereas clear phenol extracts may retain their same precipitating fraction for several months. "Double checked" pipettes were used through-

TABLE III

PRECIPITIN TESTS FOR THE FINAL DETERMINATION OF ANTIGENIC CONTENT OF VIRUS EXTRACTS

Tube No.	Cc. antiserum 1:50	Cc. antigen dilution	Phenol-saline	Precipitate*
1	0.3	0.30	0.00	+
2	0.3	0.27	0.03	+
3	0.3	0.24	0.06	+
4	0.3	0.22	0.08	+
5	0.3	0.18	0.12	+
6	0.3	0.16	0.14	+
7	0.3	0.14	0.16	±
8	0.3	0.13	0.17	o
9	0.3	0.12	0.18	o
10	0.3	0.11	0.19	o
11	0.3	0.10	0.20	o
12	0.3	0.00	0.30	o
13	0.0	0.30	0.30	o

* See Table I, footnote *.

out the experiments and the antigens were diluted with phenol-saline solution in a concentration equal to that employed in the preparation of the antigens, in order that the same amount of phenol and saline should be present in all dilutions.

Reading of results. For convenience a *unit of antigen* was defined as the smallest amount which, under a given set of conditions described above, will react to give a "slight" precipitate as defined. The amount of antigen contained in the last tube to give a positive reaction was regarded as *one unit* in strength and from this volume the number of units present in 0.3 of a cc. could be readily computed. Multiplying the dilution of antigen by the number of units present in 0.3 of a cc. gave the total number of units contained in the undiluted extract (Table IV). For example, if a

TABLE IV

ESTIMATION OF THE NUMBER OF ANTIGENIC UNITS IN THE UNDILUTED VIRUS EXTRACT

Tube No.	Cc. antigen dilution x	No. units in 0.3 cc. dilution x*
1	0.30	1.00
2	0.27	1.11
3	0.24	1.25
4	0.22	1.36
5	0.18	1.67
6	0.16	1.88
7	0.14	2.14
8	0.13	2.31
9	0.12	2.50
10	0.11	2.73
11	0.10	3.00

* Multiply by dilution x to obtain number of units in undiluted virus extract.

TABLE V
QUANTITATIVE RELATION BETWEEN ANTIGENIC CONTENT AND VIRUS CONCENTRATION OF TOBACCO VIRUS EXTRACT

History of virus extract				Comparison of antigenic content and virus concentration							
Paired virus extracts, No.	Date of preparation of extract	Date of original antigenic determination	Date of plant inoculation	No. of anti-genic units*	Virus extracts adjusted by dilution to equal antigenic content						
					Dilution of inoculum	No. of plants inoculated	Av. No. lesions per half plant	Mean No. lesions per half leaf	Mean difference in No. of lesions	S. D. mean difference	Mean difference S. D. mean difference**
1	June, 1932	Nov., 1932	July, 1933	88	1:22	16	66	11.72	1.30	0.86	1.51
2	May, 1932	June, 1933		88	1:22		74				
3	June, 1932	Nov., 1932	Mar., 1933	40	1:20	9	61	10.47	0.61	0.88	0.69
4	June, 1932	Nov., 1932		32	1:16		65				
5	Oct., 1932	Nov., 1932	Mar., 1933	14	1:7	10	72	11.38	1.15	0.83	1.39
4	June, 1932	Nov., 1932		32	1:16		65				
6	Oct., 1932	Nov., 1932	Mar., 1933	32	1:16	10	43	8.12	1.80	0.77	2.34
4	June, 1932	Nov., 1932		32	1:16		54				
7	Nov., 1933	Nov., 1933	Nov., 1933	520	1:260	10	48	8.38	0.75	0.72	1.04
8†	Nov., 1933	Nov., 1933		65	1:32.5		53				
9	Nov., 1933	Nov., 1933	Nov., 1933	260	1:130	10	94	17.00	2.63	1.34	1.96
10	Nov., 1933	Nov., 1933		130	1:65		110				
11	Nov., 1933	Nov., 1933	Nov., 1933	130	1:65	10	114	17.52	2.93	1.35	2.17
10	Nov., 1933	Nov., 1933		130	1:65		96				
8	Nov., 1933	Nov., 1933	Nov., 1933	65	1:32.5	10	104	16.34	1.98	0.95	2.08
10	Nov., 1933	Nov., 1933		130	1:65		92				

* Precipitin tests with virus extracts Nos. 1 to 6 were made at 37° C. for one hour, and Nos. 7 to 11 at 56° C. for two hours.

** A difference was considered significant when this ratio exceeded 2.39, giving odds equal to or in excess of 50:1.

† Virus extracts Nos. 8 to 11 are dilutions of No. 7. This fact was unknown at the time the above results were obtained. Nos. 7 to 11 were treated as separate antigens.

virus extract gave a positive precipitin reaction at a 1:32 dilution and a negative reaction at 1:64 (Table II), the final precipitin tests were set up according to the protocol in Table III, using decreasing amounts of dilution 1:32. Assuming that No. 6 is the last tube in which a perceptible precipitate is formed, then since 0.16 of a cc. of a 1:32 dilution of the virus extract present in this tube contains one unit of antigen, 0.30 of a cc. would contain 1.88 (Table IV), and the original virus extract would have an antigenic content of 1.88×32 , or a total of approximately 60 units. In comparing the initial antigenic content of two 10-gram lots of tissue, extracted with different volumes of saline solution, a correction for the difference in the dilution of the original juice was necessary. Unless otherwise stated, the antigenic content of a preparation has been given in terms of the prepared extract, irrespective of the amount of saline used in extraction.

Great care should be exercised in reading the precipitin tests in order to eliminate the discrepancies which may arise from non-standardized methods. A few details concerning the reaction and the technique employed by the writer may prove of some value to those wishing to repeat the experiments. In a series of tubes in which a concentrated antigen was used, those containing the lower dilutions might show a cloudiness but no floccules, while the succeeding tubes might show definite flocculation. This phenomenon is attributable to an excess of antigen and is frequently observed in serum reactions where an excess of either antigen or antibody may inhibit the reaction (34, p. 177). The tubes immediately following those with the cloudy contents should have large floccules which tend to settle out in the bottom of the tubes upon standing, leaving a clear supernatant fluid. As the antigen is further diluted, the floccules become smaller and remain suspended in the liquid, until the tube giving a slight precipitate (see Table I, footnote*) contains a few, fine, colorless flakes in suspension visible only when the tube is lighted from below and held against a black background. The writer uses a box, especially designed to give uniform lighting conditions and described elsewhere (3). A few moments after placing the tubes in the holders the heat from the two Mazda bulbs (15 watt, 120 volt) causes a circulation of the tube contents, which is most helpful in reading the reaction in tubes in which the precipitate has settled out in the bottom. The results are recorded as soon as the circulation begins. With experience the recording of results consistent with the limits of experimental error will be readily acquired, but the inexperienced person would do well to repeat the tests until the results are duplicated.

Results. The antigenic content of 11 separate tobacco virus extracts was determined and found to range from 14 to 520 units (Table V).

DISCUSSION

It was thought at first that it might be necessary to standardize the antiserum since the titer of precipitin varies with different antisera. Preliminary titrations with eight antisera have shown that the two having the greatest difference in titer varied about 90 per cent in potency, as determined by the ability of the antisera to react in increasing dilution with a given concentration of antigen. Nevertheless both antisera in a dilution of 1:50 gave the same end point for the reaction when titrated with decreasing dilutions of the same antigen. In the case of antisera falling within the range of potency stated above and employed in these tests, the 1:50 dilution of antiserum chosen for routine procedure seems to provide sufficient antibody to induce the reaction and does not constitute a large enough excess to inhibit the reaction. The fact that precipitating antisera of different titer may give the same end point for the reaction when tested with successive dilutions of a given antigen at the same dilution of antiserum has been reported (6).

Reference to Table V, footnote*, will show that the precipitin tests were run at different temperatures. The lower temperature was employed in the earlier experiments, but later a higher temperature with a longer period of incubation proved more satisfactory. Different antigens exhibit differences in the rate of reaction with the antiserum and this discrepancy was minimized by the acceleration of the reaction at the higher temperature and the more advanced end point obtained by increased incubation at a higher temperature. The solubility of the precipitate at the higher temperature, a difficulty often encountered in the precipitin reaction (32), was not evident but it remains to be shown whether or not a longer period of incubation at a lower temperature would in the end provide more suitable conditions than incubation for a shorter period at some higher temperature.

It is evident that altering the conditions of the precipitin tests so that the end point of the reaction is more advanced results in a variable unit of antigen, as defined for any given set of conditions. The further the reaction is carried, the smaller the unit of antigen. For practical purposes, a satisfactory comparison of the relative antigenic content of two extracts can be made at either temperature provided the conditions are constant for a given set of titrations. For the comparison of an indefinite number of antigens any given extract or its equivalent can be set up as a standard. If the titrations are carried out at long intervals the "standard" extract should be retested before use under the original set of conditions in order to detect any change that might have occurred during storage of the antigen, that would influence the serologic reaction.

An examination of Table V shows the wide range of antigenic units covered by the various extracts. As explained in Table V, footnote †,

virus extracts Nos. 8 to 11 are dilutions of the stock extract No. 7. Since this particular virus preparation was extracted elsewhere, details as to the method of extraction other than the freezing of the tissue are not known. It is possible that no water or only a small amount in proportion to the tissue was used during the extraction of the virus, whereas five volumes of 0.85 per cent saline to one part of tissue (green weight) were employed in the extraction of No. 2, having an antigenic content of only 88 units. The higher temperature and longer period of incubation used in determining the antigenic titer of the former extract resulted in units of unequal value for the two extracts, those in No. 7 being smaller than those in No. 2. In the case of extracts Nos. 2, 3, and 4, the tissue was taken from the same group of plants and 0.85 saline was added to one part of tissue in the proportion of 5:1, 7:1, and 8:1, respectively. Extract No. 1 was prepared from the roots of a separate group of plants that had been inoculated two weeks previously. The proportion of saline to tissue used in the extraction was 5:1. Extracts No. 5 and No. 6 were made from different parts of another group of plants, inoculated at the corresponding leaf position on each plant two weeks prior to the preparation of the antigens. The tissue extracted for No. 5 represents the leaves fifth in position above the one inoculated, whereas the tissue in No. 6 was obtained from the seventh leaf above the one inoculated in every case. In both instances saline was added in the proportion of one part of tissue to ten parts of saline. A consideration of the details involved in the preparation of the virus extracts explains the wide range of antigenic units recorded in Table V.

Having determined the antigenic content of the various virus extracts by the serum-precipitin method, in order to determine whether or not a quantitative relation existed between *antigenic content* and *virus concentration*, it was necessary to resort to a biologic method. If there were no close relation then there would be no justification for recommending the serologic method as a substitute for any biologic method in the determination of virus concentration. To test the possibility of such a relation the 11 extracts, ranging in antigenic content from 14 to 520 units, were inoculated separately on susceptible plants and the virus concentration of each was determined by its ability to infect. The local lesion method, first described by Holmes (15) was employed for this purpose. Certain modifications, which will be fully described, were adopted.

DETERMINATION OF VIRUS CONCENTRATION OF EXTRACTS

Method of plant inoculation. The plant selected for inoculation with the extracts was *Nicotiana glutinosa* L. the use of which was first recommended by Holmes (15) as a valuable method for measuring virus concentration. Holmes observed that there was a relationship between the virus concentration of the inoculum and the number of local lesions developing in the

inoculated leaves. Samuel and Bald (29), experimenting with the local lesion method, have suggested certain modifications in Holmes' routine procedure, such as the use of selfed plants to lessen the wide variation in susceptibility frequently encountered among groups of crossed plants. These authors likewise recommended the comparison of two extracts by inoculation on opposite halves of the same leaves rather than on the whole leaf surface of different plants, thus enabling the investigator to attribute differences in the number of resultant local lesions chiefly to differences in virus concentration, and not partly attributable to variation in the susceptibility of individual plants. Statistical methods for evaluating the results of inoculations were also advocated by Samuel and Bald. Caldwell (5) has employed Holmes' local lesion method in the study of aucuba mosaic and has described a method for the determination of the number of infectious particles present in a given virus extract, estimated from the number of local lesions produced by inoculating a definite volume of virus extract. In the present experiments selfed plants and the half leaf method of inoculation were used, but the volume of inoculum was not controlled. Cheesecloth thoroughly wet with virus extract was used for the inoculation of the plants and the leaf surface was rubbed once. At the completion of the inoculation of from 9 to 16 plants, the entire leaf surface was washed with running water according to the method of Holmes. The plants were trimmed to six leaves each. Right and left halves of the leaves, beginning with the youngest, were alternately inoculated with the same extract. In the case of a group of plants to be used for the same two extracts, the right half of the youngest leaf was inoculated first in the first half of the group, while the order was reversed in the second half. Samuel and Bald reported a consistent difference in the number of resultant lesions between the right and left halves of leaves inoculated with the same extract (29). These investigators recommended inoculating with one extract on the left half of all the leaves of half the plants and on the right half of the leaves on the other half of the plants. The remaining uninoculated halves of the leaves were then inoculated with the second extract, thereby offsetting any advantage that might accrue from placing one extract on the right or left halves exclusively. The present experiments were designed to minimize further experimental error.

The inoculum was never employed as a whole juice extract or in a series of dilutions but was used in a single dilution designed to give the most accurate measurement of virus concentration. From an inspection of a typical dilution curve such as that represented in Figure 1, showing the relation between virus concentration, expressed in terms of antigenic units, and numbers of local lesions produced on inoculated leaves, the slope of the curve indicates that as the concentration of virus increases the number of resultant lesions approaches a limit. It would, therefore, be

more difficult to distinguish between a difference in the concentration of two extracts falling within the range of the slope B to C than to show the same percentage difference between virus extracts falling within the range A to B. Reference to Table VI will show the results of comparing paired virus extracts covering a wide range of concentration but with the same percentage difference between the pairs. A comparison of pairs of these extracts at varying intervals on the slope ranging from 0.45 to 8 units gives odds for significance, obtained from the ratios in the last column,

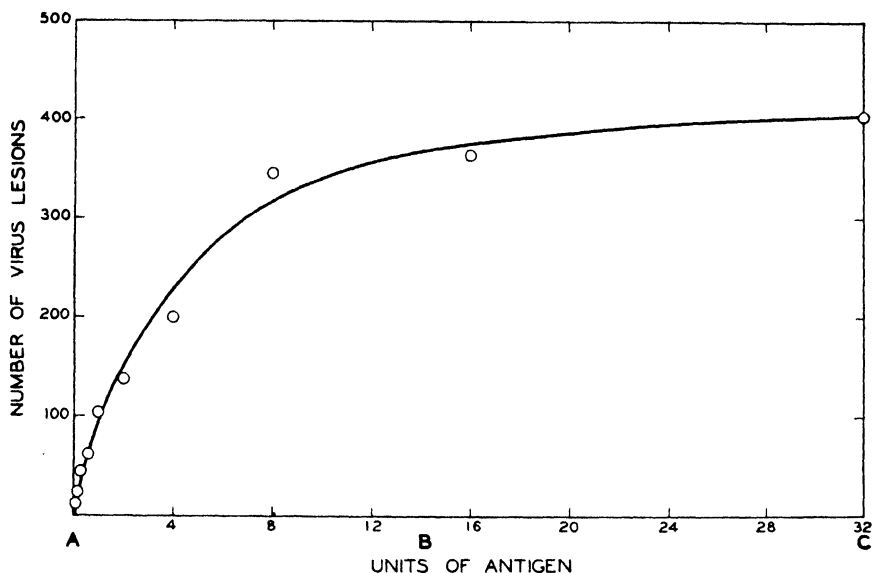


FIGURE 1. A dilution curve showing the relation between the concentration of Tobacco virus 1 and the number of local lesions per plant produced on *Nicotiana glutinosa*. The concentration of virus is expressed in terms of antigenic units.

in excess of the odds obtained by the comparison of extracts of the same percentage difference in the more concentrated range of 14.4 to 57.6 units. The odds reach a maximum in the range of 1.80 to 3.60 antigenic units and are consistently greater at this range. From the maximum the odds fall off in both directions but more rapidly in the direction of increasing concentration where the curve flattens. As the result of this observation all extracts were diluted prior to inoculation to a concentration represented within the range A to B. In the comparison of paired extracts, dilutions were made on the basis of their respective antigenic content, as indicated in Table V, so that either two or four units were present in the final dilution. The two extracts, diluted to equal antigenic content, were then inoculated on opposite halves of the same leaves and the numbers of lesions developing within four to six days were counted.

TABLE VI
COMPARISON OF PAIRED TOBACCO VIRUS EXTRACTS COVERING A WIDE RANGE OF CONCENTRATION BUT WITH 100 PER CENT DIFFERENCE
BETWEEN PAIRS; NICOTIANA GLUTINOSA

Exp. No.	Half plant units					Half leaf units				
	No. anti-genic units in diluted virus extract*	No. plants inoculated	Mean No. lesions per half plant	Difference in No. lesions	S. D. of difference	Difference in No. lesions S. D. difference**	Mean No. lesions per half leaf†	Mean difference in No. lesions	S. D. mean difference	Mean difference S. D. mean difference**
1	0.45 0.90	9	15 21	6	4.96	1.21	2.94	1.02	0.38	2.68
2	0.45 0.90	9	31 58	27	11.22	2.41	7.54	4.66	1.06	4.40
3	0.45 0.90	9	76 116	40	17.12	2.34	16.01	6.76	1.13	5.98
4	0.90 1.80	8	48 80	32	15.68	2.04	10.69	5.38	1.46	3.68
5	0.90 1.80	9	51 121	70	25.90	2.70	14.28	11.67	2.19	5.33
6	1.80 3.60	9	32 77	45	15.79	2.85	9.06	7.54	0.96	7.85
7	1.80 3.60	7	40 80	40	10.35	3.86	9.95	6.62	1.01	6.55
8	1.80 3.60	9	94 146	52	28.86	1.80	19.96	8.70	1.69	5.15
9	1.80 3.60	9	98 162	64	45.67	1.40	21.74	10.67	2.05	5.20

TABLE VI (Continued)

Half plant units										Half leaf units			
Exp. No.	No. anti-genic units in diluted virus extract*	No. plants inoculated	Mean No. lesions per half plant	Difference in No. lesions	S. D. of difference	Difference in No. lesions S. D. difference**	Mean No. lesions per half leaf†	Mean difference in No. lesions	S. D. mean difference	Mean difference S. D. mean difference**			
10	4.00 8.00	9	56 86	30	15.35	1.95	11.86	4.94	0.85	5.81			
11	4.00 8.00	9	62 76	14	14.69	0.95	11.53	2.31	1.22	1.89			
12	4.00 8.00	9	74 93	19	22.74	0.84	13.98	3.15	0.91	3.46			
13	4.00 8.00	9	100 154	54	25.40	2.13	21.16	8.98	1.99	4.51			
14	14.40 28.80	9	121 182	61	36.41	1.68	25.23	10.13	2.47	4.10			
15	14.40 28.80	9	223 325	102	49.29	2.07	47.44	17.73	3.46	5.12			
16	14.40 28.80	9	470 484	14	92.49	0.15	79.45	2.39	4.27	0.56			
17	28.80 57.60	9	192 235	43	30.48	1.41	36.29	7.23	2.83	2.55			
18	28.80 57.60	9	396 470	74	54.56	1.36	77.92	13.28	4.97	2.67			
19	28.80 57.60	7	392 602	210	138.00	1.52	91.59	38.66	9.36	4.13			

* In experiments Nos. 1 to 9, and 14 to 19, the same virus extract was used; in Nos. 10 to 13 a different extract was employed. Their relative concentrations were determined both by the serum-precipitin method and by inoculation on *Nicotina glutinosa*.

** See Table V, footnote **.

† Not all plants had six leaves. This accounts for apparent discrepancies in mean number of lesions per half plant and half leaf.

Statistical treatment of results. Samuel and Bald emphasized the advantage to be gained by handling the data statistically. These authors have demonstrated the greater significance derived from a comparison of two extracts by inoculating them on opposite halves of the same leaves, rather than on whole leaves of separate plants (29, p. 81). The main advantage accruing from the use of corresponding halves of leaves is the elimination of a difference in plant susceptibility as a factor, which in turn permits a pairing of items in the statistical treatment with a consequently greater significance attributable to the results. The method employed in the present experiments gives an added significance to the results. An example of the procedure is given in Table VII. Student's method

TABLE VII

STUDENT'S METHOD FOR COMPARISON OF PAIRED TOBACCO VIRUS EXTRACTS ON BASIS OF HALF LEAF UNITS; NICOTIANA GLUTINOSA

Leaf No.	No. lesions on opposite halves of same leaf		Difference in No. lesions (A - B)	(A - B) ²	$\frac{[\sum (A - B)]^2}{n}$	$\frac{\sum (A - B)^2 - [\sum (A - B)]^2}{n}$	$\frac{S}{n(n-1)}$	S.D. mean difference
	Extract A	Extract B						
1	1	4	-3	9				
2	1	4	-3	9				
3	5	4	1	1				
4	9	9	0	0	(30) ²	394.00		
5	15	8	7	49		112.50		
6	22	8	14	196	8			
7	4	1	3	9	= 112.50	281.50 = S	5.03	$\sqrt{5.03}$
8	11	0	11	121				= 2.24
			30	394				

(9, p. 112) was followed but instead of pairing half plants each half leaf was compared with the opposite half. In 42 separate experiments in which Student's method was applied to the same data using a half plant as a unit and then a half leaf as a unit, 30 out of 42 of the tests showed greater odds for significance when half leaf units were used. The results of inoculation experiments (Tables VI and VIII) record the same data computed according to Student's method outlined in Table VII and referred to as the method of "half leaf units," and a method involving a comparison of the difference in the mean number of lesions per half plant produced by each of two extracts with the standard deviation of the difference. The latter method has been designated as the method of "half plant units" and for the sake of comparison with the other method the ratio between the difference in the mean number of lesions per half plant and the standard deviation of the difference has been computed. The greater significance in favor of the treatment by half leaf units is apparent from a glance at the corre-

TABLE VIII
COMPARISON OF PAIRED TOBACCO VIRUS EXTRACTS OF VARYING PERCENTAGE DIFFERENCES IN CONCENTRATION; NICOTIANA GLUTINOSA

Half plant units										Half leaf units			
Exp. No.	No. anti- genic units in diluted virus extract	Per cent difference in concen- tration	No. plants inocu- lated	Mean No. lesions per half plant	Differ- ence in No. le- sions	S. D. difference	Difference in no. lesions S. D. dif- ference	Mean No. lesions per half leaf**	Mean dif- ference in No. le- sions	S. D. mean dif- ference	Mean dif- ference in No. lesions S. D. mean difference		
1	3.00	150	9	36 56	20	10.50	1.90	7.70	3.26	0.78	4.18		
	7.50												
2	3.00	80	16	43 61	18	8.21	2.19	8.59	3.00	0.67	4.48		
	7.50												
3	1.80	50	9	33 82	49	14.41	3.40	9.61	8.26	1.21	6.83		
	3.24												
4	3.00	50	16	37 92	55	10.44	5.27	10.76	9.03	0.83	10.88		
	4.50												
5	3.00	50	9	72 83	11	7.32	1.50	12.94	1.72	0.86	2.00		
	4.50												
6	3.00	33½	16	64 76	12	6.28	1.91	11.68	2.00	0.64	3.13		
	4.50												
7	3.00	25	9	39 46	7	8.25	0.85	7.12	1.13	0.59	1.92		
	4.50												
8	3.00	33½	16	42 51	9	6.71	1.34	7.73	1.54	0.48	3.21		
	4.50												
9	4.00	50	10	59 74	15	13.09	1.15	11.10	2.40	0.78	3.08		
	6.00												
10	3.00	33½	9	52 64	12	9.95	1.21	9.66	1.94	0.86	2.26		
	4.00												
11	2.88	25	16	104 118	14	15.09	0.93	18.45	2.33	1.15	2.03		
	3.60												

* See Table V, footnote **.

** See Table VI, footnote †.

sponding columns of the ratios (Tables VI and VIII). The computation used by the present author in the method of half plant units is not the most efficient treatment of the data. Half plant units allow an application of Student's method as employed by Samuel and Bald. Whole plant units do not permit the pairing of items utilized to so good an advantage in Student's method. The comparison was made in order to show that the value of such a modification in technique may be lost if the statistical treatment is not used to the best advantage. In comparing virus extracts of known

TABLE IX

COMPARISON OF PAIRED TOBACCO VIRUS EXTRACTS OF IDENTICAL CONCENTRATION BY HALF LEAF UNITS; *NICOTIANA GLUTINOSA*

No. antigenic units in di- luted virus extract	No. plants inocu- lated	Mean No. lesions per half leaf	Mean difference in No. of lesions	S. D. mean differ- ence	Mean differ- ence in No. lesions * S. D. mean difference
3 3	16	12.57	0.22	0.63	0.35
3 3	16	5.52	0.14	0.35	0.40
3 3	16	5.70	0.36	0.44	0.82
3 3	16	6.57	0.60	0.43	1.40
4 4	10	7.92	0.47	0.67	0.70
4 4	9	18.44	0.59	1.42	0.42

* See Table V, footnote**.

percentage differences in concentration (Table VIII) the method of half leaf units can be relied upon to distinguish between extracts differing 50 per cent in concentration, provided a sufficient number of plants is inoculated, whereas the method of half plant units in which a less efficient statistical treatment of the data is made fails in every instance. It is also clearly shown that the significance improves with an increase in the number of inoculated leaves. From the ratio obtained by comparison of paired extracts differing $33\frac{1}{3}$ and 25 per cent in concentration (Table VIII, Exp. Nos. 6 and 7) it is not unreasonable to suppose that an appreciable increase in the number of plants inoculated would provide sufficient odds for significance. Table IX provides a treatment of data obtained from the inoculation of the identical dilution used as separate inoculums by the

method of half leaf units. Six separate experiments with 12 paired extracts gave insignificant odds for a difference in every case. Student's method dealing with half leaf units was adopted as routine procedure in the evaluation of the data obtained by the local lesion method. A detailed statistical analysis of the local lesion method will appear simultaneously with this article (33).

Results. When the 11 virus extracts were diluted so that they contained an equal number of antigenic units and were then inoculated in eight different pairs on opposite halves of leaves of *Nicotiana glutinosa*, no significant difference was demonstrable in seven of the pairs and a barely significant difference in the case of one pair (Table V).

It is worthy of note that when virus extract No. 1 was inoculated on plants one year after preparation and was compared with No. 2 inoculated two months after extraction, no significant difference was demonstrable when the antigenic content of the two extracts was used as the basis for comparing the active virus concentration of the two.

DISCUSSION

In the experiments described above only one type of virus extract has been tested, viz., saline extracts of green tissue with the addition of phenol. No attempt has as yet been made to test the quantitative relation between antigenic content and the virus concentration of extracts in which a partial or total inactivation of the virus has been induced, such as Matsumoto and Somazawa claim for formalinization of extracts (21). No reagents have been used that are known to interfere with the precipitin reaction or falsify the measurement of active virus on the plant, since the objective of the experiments is to test the quantitative relation between antigen and active virus concentration. Consequently, conditions most favorable for both reactions were sought.

If the quantitative relation fails with an unknown preparation, the likelihood of some condition unfavorable to the serum or plant reaction should be considered. Until further information is available, therefore, it is essential before arriving at any conclusion regarding the virus concentration of an unknown antigen on the sole basis of the serum-precipitin reaction, to test by plant inoculation the relation between antigen and active virus by comparing the unknown with a standard solution of known concentration according to the method previously described for the comparison of two extracts. The standard virus extract can be obtained by the serologic technique.

The value of the serum reaction would be greatly enhanced if it could be shown conclusively that specific precipitin reacts with active and inactivated virus, or that the reactive substance in virus extract is derived directly from virus and hence a logical indicator of virus content. Deter-

mining virus concentration by means of infectivity tests is a measure only of active virus, whereas serum reactions as generally employed in other fields are independent of the vitality of the reactive substance. Bacteria for example are agglutinated either dead or alive; toxins are flocculated in specific antitoxin mixtures, either as anatoxins (detoxified toxins) or potent toxins. The danger of carrying this analogy too far lies in the possibility that with utterly unknown entities, such as filterable viruses, pathogenicity and antigenicity may not be independent of each other. This question affords an interesting subject for further investigation and the importance of this phase of the problem merits careful consideration. An investigation of this nature would appear to be greatly benefited by precise quantitative determinations both of the antigenic content and virus concentration of a given sample of mosaic juice before treatment and again after treatment with various reagents such as formalin which apparently affects one property, in this case infectivity, more than the other property, antigenicity.

An illustration of a preliminary experiment in this field follows. The amount of precipitating substance in a sample of tobacco virus extract was determined by titration with specific antiserum. The active virus concentration was then estimated by plant inoculation, dividing the sample equally and treating the two lots as separate extracts. The results of the infectivity tests showed that the two lots were indistinguishable as to virus content. Part of the sample was then subjected to a strongly alkaline reaction (pH 8.4) by the addition of a buffer. After allowing the alkaline extract to stand for several days, the amount of precipitable substance was again measured quantitatively by the serum-precipitin reaction and the virus concentration was determined by plant inoculation and the results were compared with those from the untreated sample. A loss of approximately one-third had occurred in the amount of precipitating substance whereas a loss of about four-fifths in the concentration of active virus had taken place. Certain factors should be considered here in interpreting the results, such as the addition of electrolyte to the virus extract and its possible effect on the precipitin reaction. Conditions unfavorable to the successful inoculation of the plant should also be investigated, such as the hydrogen ion concentration of the inoculum. The extract used for inoculation here was tested both at pH 8.4 and after neutralization, but it was not possible to increase the infectivity of the inoculum by adjustment of the reaction.

The writer is aware of the fact that virus extract may be quite a complex antigen consisting of a combination of several different antigenic substances which in turn would be capable of producing a corresponding variety of precipitins in antiserum. Apparently, if this be the case with tobacco virus extract, it does not interfere with the estimation of antigen,

bearing a relation to the virus concentration, by the antigen-dilution method herein described.

By comparison with the results obtained by the inoculation of known concentrations of virus extracts on *Nicotiana glutinosa* (Table VIII) it has been possible to ascertain approximate percentage differences in concentration demonstrable by the biologic method. Since seven of the eight pairs of extracts used for testing the quantitative relation between antigenic content and active virus concentration (Table V) were inoculated on from nine to ten plants only, it would be unwise to assume that the extracts did not exceed 50 per cent difference in concentration. Such was doubtless the case between extracts Nos. 1 and 2 where 16 plants were used for inoculation. The other seven pairs of extracts may be regarded as not exceeding a difference of 80 per cent in concentration. As the biologic method is employed at the point where it approaches the sensitivity obtainable by the serologic method, it will be interesting to note how closely the quantitative relation between antigen and virus is maintained. Should the quantitative relation between antigen and active virus prove to be different as the comparison between the two methods is drawn more sharply, further experimentation would be required before any conclusions could be drawn as to the lack of the identity of antigen and virus. To reiterate former statements, the biologic method is a measure of active virus solely while the serologic method may be estimating the active and inactive virus content of the extracts. Considering this possibility it seems the more remarkable that the quantitative relation between antigen and active virus has proved as close as shown by the results.

SUMMARY AND CONCLUSIONS

1. The literature dealing with the immunologic reaction in relation to filterable viruses of plants is reviewed and a precipitin reaction is recommended as a qualitative test in the detection of masked carriers in the identification of new hosts and in the classification of plant viruses.

2. *Solanum melongena* L. var. Hangchow Long, *S. sisymbriifolium* Lam., *Physalis peruviana* L., *Capsicum minimum* Blanco, *C. frutescens* L. var. Pimiento, all affected with tobacco mosaic virus, and *Nicotiana tabacum* L. var. Turkish, affected separately with aucuba mosaic and attenuated tobacco mosaic, are found to yield extracts giving a positive precipitin reaction with antiserum to Tobacco virus 1. Extracts of *Asclepias curassavica* L., *Datura stramonium* L., and *Solanum melongena* L. var. Black Beauty affected with mosaic diseases other than tobacco mosaic are found to react negatively with antiserum to Tobacco virus 1.

3. A method for determining the antigenic content of saline extracts of Tobacco virus 1 to which phenol has been added is described in detail.

4. Certain modifications in Holmes' local lesion method employed in

the estimation of the active virus concentration of these extracts are described. The use of inoculum at a concentration within the most precise range on the dilution curve is stressed.

5. From the results of inoculations of known concentration on *Nicotiana glutinosa* L. it is shown that a difference in the concentration of virus extracts as small as 50 per cent is demonstrable when 16 plants are used. It is pointed out that by increasing the numbers of inoculated plants, as small a percentage difference as 25 per cent should be demonstrable.

6. A quantitative relation is shown to exist between the antigenic content and the active virus concentration of 11 separate extracts compared in eight different pairs. In seven out of eight of these pairs the concentration of virus probably does not vary more than 80 per cent and in the case of the remaining pair, probably less than 50 per cent.

7. The conclusion is drawn that the serologic technique is valuable as a rapid and accurate means of determining the relative virus concentration of saline mosaic extracts to which phenol has been added.

8. The results offer no evidence thus far to conflict with the contention that the specific antigenic material in Tobacco virus 1 extract may be virus itself.

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A STATISTICAL STUDY OF THE LOCAL LESION METHOD FOR ESTIMATING TOBACCO MOSAIC VIRUS

W. J. YODEN AND HELEN PURDY BEALE

INTRODUCTION

In 1929 Holmes (5) published a method for measuring the concentration of mosaic virus. This method depends upon the appearance of local lesions on *Nicotiana glutinosa* L. leaves after they have been rubbed with a piece of cheesecloth soaked in the virus extract. The number of lesions serves as a measure of the concentration of the virus. Holmes (5, p. 47) proposed the following procedure. "*N. glutinosa* plants are grown in four-inch clay pots until flower buds begin to appear. At this stage at least five leaves on each plant are of good size. These five leaves are used for the inoculation; and for convenience in manipulation all the remaining leaves of the plant, as well as the growing point, are pinched off. This leaves a sturdy stem supporting five large leaves. Virus from any source to be tested is now taken up on a small piece of white cheesecloth and rubbed once firmly but gently over the entire upper surface of the five leaves. A full stream of tap water is used to wash away excess virus at once after this inoculation." On page 51 Holmes gives a curve showing the "probable errors of single observations for test plants having numbers of lesions between 0 and 400." It is evident that this author took the sum of all the lesions appearing on the five leaves and used this plant total as his unit.

Subsequently Samuel and Bald (6) modified Holmes' procedure. This adaptation enhances the accuracy of the comparison of two virus preparations. Briefly the method consists in rubbing the right half of each leaf with the first preparation and the left half with the second preparation. After this has been done on five plants the process is repeated on five more plants, placing the first preparation on the left half. Samuel and Bald obtain the total number of lesions on the five right halves and compare this with the total on the five left halves. In this way they accomplish a comparison for every plant. The mean of the ten differences and its standard deviation are used to interpret the relation between the two preparations. The unit in these calculations is the total from five half leaves or half a plant.

Neither of the two papers referred to contains any extensive statistical examination of the data. The authors used *totals* of their fundamental experimental units (the leaf and the half leaf). Although this decreases the arithmetical labor there is the possibility that valuable information is lost by this step. The senior author has examined certain of the data obtained

by Beale(1) in this laboratory. The data consist of 39 tests involving 529 plants and 93,822 lesions. The results of this investigation show that the interpretation of the data by means of the analysis of variance simplifies the work of separating the individual effects of several variables operating simultaneously in a given experiment. It has also been possible to gain some additional knowledge about the experimental procedure. This information provides a sound basis for further modifications of the technique. This proposed procedure is less complicated than that used by Samuel and Bald and makes it easier to intercompare a number of solutions.

The statistical procedure will be discussed in some detail to facilitate its use by those unfamiliar with it. It is not feasible to publish all the original counts used in these computations. Care has been taken, however, to tabulate for all the data the final stages of the computations in order that the reader may examine the basis for the deductions made here.

IMPORTANT FACTORS IN THE LEAF SPOT METHOD

The biometrical study given in detail in this paper affords new evidence regarding factors previously recognized as important in the leaf spot method. It appears that different plants show varying degrees of susceptibility to infection by the virus. One way this factor can be eliminated is to arrange the experiment so that each virus preparation is applied to at least one leaf on each plant. This is not provided for in Holmes' technique. The work of Samuel and Bald indicates that such a procedure is feasible.

A careful study was made of the effect of leaf position on the plant. It is not possible to state a simple rule describing this phenomenon. Depending upon the particular group of plants employed, the gradient may take several forms. The top leaf may be the most susceptible with succeeding leaves showing lower counts in order of their position on the plant. In other cases the exact reverse is true. Cases are also presented showing that the middle leaves may have the largest counts with decreasing counts obtained from the top and bottom leaves. At other times the middle leaves show a minimum. Several examples of these cases are shown in Figure 1. It must be understood that this does not mean that the effect of leaf position can be ignored. Within any group of plants used in a given experiment there will be found a strong correlation of the count with the leaf position. This correlation should be utilized in estimating the standard deviation of the measurements. In general the reduction in the estimated error amounts to nearly one-half.

The data also permit another interesting observation. Three groups of plants (large, medium, and small) were each divided into two sets and one set from each group decapitated according to Holmes' recommendation. The analysis shows this to be a valuable feature of the experimental tech-

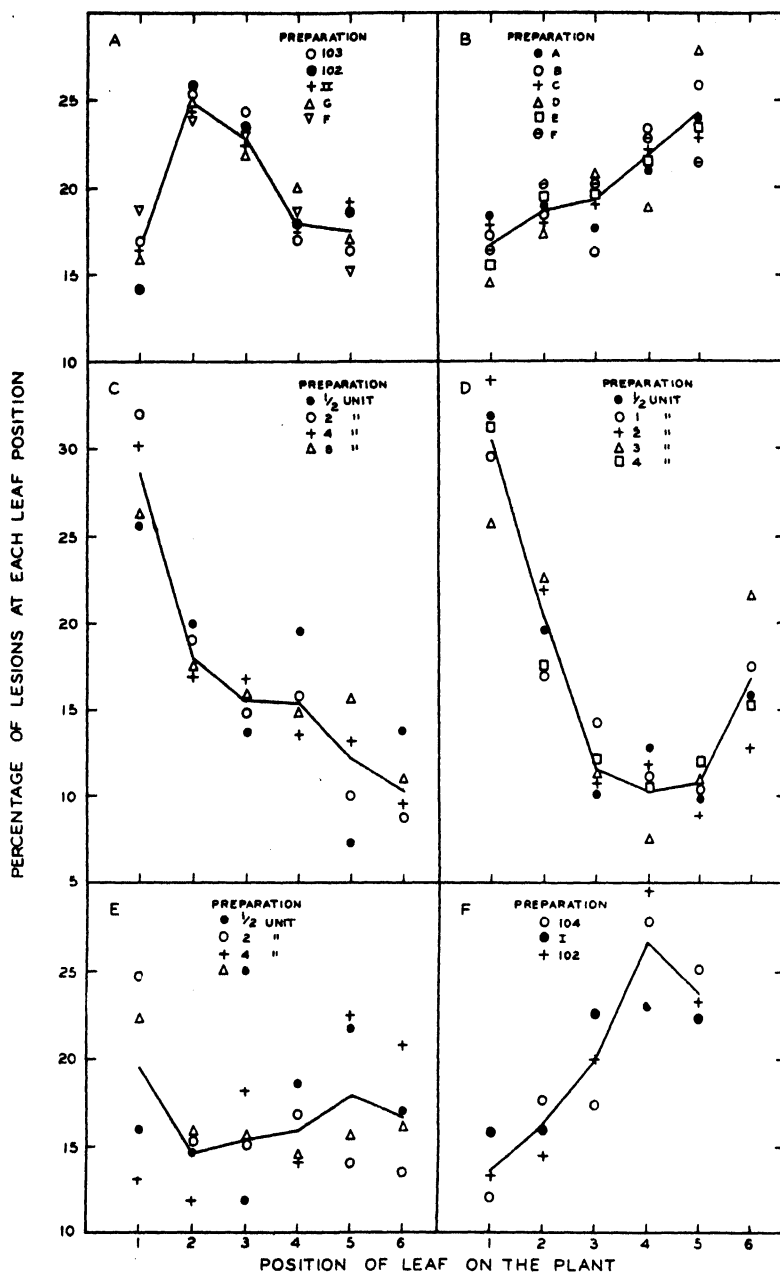


FIGURE 1. The points show the percentage of lesions found on leaves from different positions on the plant. The six sets were inoculated at different times. Within any set the several preparations tested show a marked uniformity in the fraction of the total number of lesions found at each leaf position.

nique. The decapitation resulted in diminished variation among the plants and among the leaves on any given plant.

A considerable number of the plants used in this study were inoculated by the half leaf method. Although Samuel and Bald report a difference in the counts obtained on the right and left halves there is no evidence in these experiments of such a condition. They supposed that the manner of holding the leaf accounted for the observations. Apparently this difficulty was overcome in the inoculations made in this laboratory.

Another source of error lies in the use of different pieces of cloth for applying the virus. Naturally two different pieces are used if two preparations are being compared. If the cloth introduces an effect peculiar to itself, a difference in counts obtained with two preparations may be due to the use of different pieces of cloth. It was possible to attempt to answer this question since several of the experiments were inoculated in groups of four using a fresh cloth for each group, other conditions remaining unaltered. There were available nine experiments totalling 54 sets of four plants. It was concluded that the error arising from this source was small, although the calculations suggest that four plants rubbed with different cloths vary more than four plants with the same cloth.

THE ANALYSIS OF VARIANCE APPLIED TO LESION COUNTS

Before considering the statistical procedure in detail, it will be instructive to develop the analogy between the method of laying out experimental field trials and the design of leaf spot experiments. Suppose five agricultural treatments, A, B, C, D, and E, are to be compared. If these are each replicated five times an excellent arrangement by Fisher (2, p. 246) is that of the Latin square shown below.

A	B	C	D	E
D	A	B	E	C
C	E	D	A	B
B	D	E	C	A
E	C	A	B	D

The 25 plots are so arranged that each treatment occurs once in every row and once in every column. The totals of the five plot yields in each row and each column will disclose any inhomogeneity in the experimental field. By the use of these totals it is possible to estimate and eliminate the contribution to the variation among the plot yields arising from the inequalities in the field, just as the treatment totals are used in determining whether differences exist among the treatments. If we consider each plot as a single leaf, each column a plant, and each row the corresponding leaf on

each plant the statistical procedure used in evaluating field data may be taken over bodily in evaluating the leaf spot data. Each treatment must occur equally often on each plant and on every leaf position. This simply requires that the number of plants be an integral multiple of the number of solutions being compared.

Suppose that five plants have been inoculated with the same solution and the number of lesions recorded for the twenty-five leaves. Table I shows the data obtained in one experiment. The letters A, B, C, D, E have been assigned to the leaf counts so that each letter appears on every plant and at every leaf position. The totals for the 5 A's, the 5 B's, etc.,

TABLE I
ANALYSIS OF VARIANCE APPLIED TO LESION COUNTS

Leaf position	Plant number					Total each leaf position	Sample totals
	1	2	3	4	5		
1	18(A)	25(E)	36(D)	35(C)	23(B)	137	204(A)
2	11(B)	33(A)	29(E)	43(D)	22(C)	138	140(B)
3	5(C)	44(B)	49(A)	78(E)	28(D)	204	146(C)
4	24(D)	36(C)	27(B)	50(A)	15(E)	152	207(D)
5	5(E)	76(D)	48(C)	35(B)	54(A)	218	152(E)
Totals	63	214	189	241	142	849	849

Sum of the squares of the differences of the leaf counts from the average leaf count 8352.96

Sum of the squares of the differences of the plant totals from the average plant 19570.8

Sum of the squares of the differences of the leaf position totals from the average leaf total 5896.8

Sum of the squares of the differences of the five samples from the mean sample 4324.8

Analysis of Variance

Item	Degrees of Freedom	Sum of Squares	Mean Square (Variance)	Standard Deviation	<i>z</i>
Plants	4	3914.16	978.54		0.7951
Leaf Positions	4	1179.36	294.84		0.1952
Samples	4	864.96	216.24		0.0402
Error	12	2394.48	199.54	14.126	
Total	24	8352.96			

are shown in the last column of the table. It is apparent that these five totals vary less than the five plant totals, or the five leaf totals. This is not surprising since each group of letters has had an equal opportunity to sample the plants and leaf positions. Had it been desired to test five different solutions, sets of leaves selected in this manner would greatly enhance the detection of differences among the solutions.

The letters might have been assigned in other ways and still fulfill the sampling requirement. Naturally the sample totals alter if the letters are assigned in some other order but it will be found that the average spread of the sample totals is much less than the spread of the plant totals.

An estimate of the appropriate probable error or standard deviation of the sample totals could be obtained by direct calculation from the five totals. This could be done for several different arrangements of the letters, and the average of the several estimates of the standard deviation taken. The analysis of variance is a short cut to this procedure.

Below the table are listed the arithmetical quantities required. Before these quantities are transferred to the section headed Analysis of Variance they must be divided by certain factors. The leaf is the unit throughout. The plant, leaf, and sample totals are all sums of five leaves and the sums of the squares obtained from them must be divided by five before entering under the heading Sum of Squares. The sum obtained from the 25 leaf counts constitutes the total in this column. The other sums are entered opposite the appropriate entries in the Item column, leaving temporarily blank a space opposite Error. The Error designates the variance not accounted for by plant differences, leaf position differences, or sample differences, if any, and is found by subtracting the sum of these from the total. The entries listed under Degrees of Freedom are one less than the number of quantities summed to give the squares, that for Error being found by difference. The Mean Square (or Variance) is found by dividing the Sum of Squares by the proper Degrees of Freedom. This column indicates the relative importance of the several factors in accounting for the variation among the 25 leaf counts. The Mean Squares for Plants and Leaf Positions exceed that obtained for Error, but the Mean Square for Samples is practically equal to the value opposite Error. Since the Samples were actually the same (all leaves received the same solution) there should be no contribution from this source. If Samples had not been arbitrarily assigned there would have been obtained for Error 16 Degrees of Freedom with a Sum of Squares entry of 3259.44 and a Mean Square of 203.72. In this case the Degrees of Freedom ordinarily taken by Treatments become available for estimating the error and the best estimate of the standard deviation of a single leaf is the $\sqrt{203.72}$.

The equivalence of the Mean Squares for Samples and Error is sufficient evidence that the Samples (or Treatments) were all alike. It will be of interest however to examine the difference between two Samples, in terms of the standard deviation of this difference. The standard deviation of a single leaf is 14.126, and therefore of the sum of five leaves is $14.126\sqrt{5}$. This result, multiplied by the $\sqrt{2}$ to obtain the standard deviation of the difference, gives 44.66. Even the extreme Samples do not differ by twice this amount.

It is important to have some means of evaluating the quantities listed under Mean Square. Fortunately an exact statistical criterion of these quantities is available in Fisher's function z described on page 206 of his text (2). The z values are found by looking up the natural logarithms of the

Mean Square quantities and subtracting that obtained for Error from each of the others and taking one-half of the differences obtained. On pages 224-225 of Fisher's text (2) a table of values of z is given from which in the appropriate column and row ($n_1 = 4$; $n_2 = 12$) the z value 0.5907 is obtained. The z found for plants exceeds this and the odds are greater than 20 to 1 that the plants differed in susceptibility. On pages 226-227 (2) z values for a higher level of significance are available, z in this case being 0.8443. The odds are therefore less than 100 to 1 although greater than 20 to 1 that plant differences have been established. In this experiment the leaf positions have not been shown to be significantly different in susceptibility. A larger number of plants would be required to establish this.

Additional details concerning the analysis of variance may be found in texts (2, 7, 8) as well as in recent papers (3, 4).

DETAILED EXAMPLE OF THE ANALYSIS OF VARIANCE

Table II is a copy of a specimen work sheet showing both the original data and the calculations required in the analysis of variance. In this experiment six leaves from each of eight plants were inoculated by a half leaf technique. The two treatments were alternated on the right and left halves within each plant. The total variance of the 96 counts has been subdivided into several divisions. The observed counts vary among themselves because two different preparations were applied. They also show variation because the eight plants differ in susceptibility. The totals for the six leaf positions also show great variation. The count of 452 for the 48 left halves is very close to the figure of 443 for the right halves. All these sources of variation may properly be eliminated since both treatments appeared equally often on all eight plants, in all six positions and on the two halves.

The procedure employed in Table II for calculating the column Sum of Squares is somewhat different from that used for the data in Table I. The two methods are exactly equivalent. The calculations shown in Table II avoid the arithmetical labor of obtaining differences from the several means. The quantity 13267 under Entries is the sum of the squares of the 96 half leaf counts; in other words, the original observations. This quantity, diminished by the square of the grand total divided by the number of observations, is 4923.0 and this figure is the sum of the squares of the differences of the 96 counts from their general mean. The column under Whole Plants makes use of the eight plant totals listed at the extreme right of the table. The factor 12 arises through the fact that 12 half leaves constitute a plant. Similarly 16 is the proper factor in the column Leaf Positions because there are 16 half leaves at each leaf position. The sub-totals in the body of the table are used only to facilitate the forming of

TABLE II
SPECIMEN DATA SHEET SHOWING DETAILED LESION COUNTS ON HALF LEAVES
AND COMPUTATIONS

Plant	Leaf No. 1		Leaf No. 2		Leaf No. 3		Leaf No. 4		Leaf No. 5		Leaf No. 6		Total each plant
	L	R'	L'	R	L	R'	L'	R	L	R'	L'	R	
1	4	2	9	10	4	3	5	1	4	5	6	9	62
3	7	19	16	10	4	12	9	5	3	13	26	11	135
5	21	16	31	18	7	17	7	4	3	5	19	26	174
7	20	18	18	14	3	3	5	5	2	6	3	6	103
Total	52	55	74	52	18	35	26	15	12	29	54	52	
2	L'	R	L	R'	L'	R	L	R'	L'	R	L	R'	
4	8	10	6	7	4	7	5	0	5	3	8	19	82
6	40	16	7	8	5	4	3	4	8	9	2	10	116
8	21	15	17	19	10	5	5	2	6	9	23	11	143
8	10	13	5	10	1	9	4	4	7	2	6	9	80
Total	79	54	35	44	20	25	17	10	26	23	39	49	
Leaf total	240		205		98		68		90		194		895

L and R refer to the halves of the leaves. Prime indicates half receiving four-unit preparation. The other half received three-unit preparation. $L+L'=452$; $R+R'=443$; $L'+R'=501$; $L+R=394$.

	Entries	Whole Leaves	Treatments	L. Half R. Half	Whole Plants	Leaf Positions	Half Plants
Sum of Squares	13267	24349	406237	400553	109983	159589	57239
Divide by	1	2	48	48	12	16	6
Quotient	13267.0	12174.5	8463.3	8344.9	9165.3	9974.3	9539.8
Subt.							
$(895)^2 \div 96$	8344.0	8344.0	8344.0	8344.0	8344.0	8344.0	8344.0
	4923.0	3830.5	119.3	0.9	821.3	1630.3	1195.8

Analysis of Variance

Item	Degrees of Freedom	Sum of Squares	Mean Square (Variance)	Standard Nat. Log. Deviation	Mean Sq.	Diff.	$\frac{1}{2}$ Diff. (z)
Treatment	1	119.3	119.3		2.4791	1.7305	0.8653*
Whole Leaves	47	3830.5	81.5		2.0980	1.3494	0.6747**
L. Half R. Half	1	0.9	0.9				
Error	46	972.3	21.14	4.598	0.7486		
Total	95	4923.0					
Whole Plants	7	821.3	117.3		2.4622	1.7136	0.8568**
Leaf Positions	5	1630.3	326.1		3.4846	2.7360	1.3680**
Interaction	35	1378.9	39.40	(6.277)			
Whole Leaves	47	3830.5					
Whole Plants	7	821.3					
Treatment	1	119.3					
Interaction	7	255.2	36.46	(6.038)			
Half Plants	15	1195.8					

* Exceeds value in Fisher's five per cent table of z.

** Exceeds value in Fisher's one per cent table of z.

the totals for the two treatments and the total 452 for the 48 left halves and the total 443 for the 48 right halves. The sum of the squares of these two totals is 400,553. The sum of the squares of 501 and 394, the two treatment totals, is 406,237. The operations in the section headed Analysis of Variance have been described in connection with Table I.

The 96 half leaves make available a total of 95 degrees of freedom. The subdivision of the degrees of freedom into several sections shows the relations between various ways of interpreting the data. Thus the total of 95 may be divided into three parts. One part with 47 degrees of freedom allows for the variation in the counts arising from differences in susceptibility of the 48 whole leaves. A second part with one degree of freedom provides for the differences in the counts introduced by the use of the two treatment solutions. The residue with 47 degrees of freedom is the best estimate of the error and is identical with that obtained by applying Student's method to the 48 pairs of half leaves. One degree of freedom from this group may be utilized to examine the relative susceptibility of the right and left halves of the leaves, leaving 46 degrees of freedom for the evaluation of the error. Since this study shows no advantage exists for either half of a leaf this degree of freedom may be included with the other 46 for estimating the error. The fraction of the variance with 47 degrees of freedom representing variation among the 48 whole leaves may be subdivided to throw light on several questions. It may be broken up giving a part with 7 degrees of freedom which discloses the variability of the plants used. The variance of this part gives the estimate of error that was necessarily used in the old technique of inoculating whole plants with one preparation. A comparison of the variance of this item with that calculated from the item designated Error shows clearly the immense advantage of the half leaf technique. A second part included in the 47 degrees of freedom assigned to leaves has 5 degrees of freedom and arises through differences in susceptibility of leaves in different positions on the plant. This is the most efficient criterion for evaluating the possible advantage of decapitation of the plant as a means of minimizing a gradient of susceptibility within the plant. The residue with 35 degrees of freedom represents the interaction of plants with leaf position. In other words it discloses whether different plants of any given set show different gradients of susceptibility. This is the item which in Table I was taken as the estimate of error in the experiment using whole leaves as the unit of inoculation. This quantity approximates closely the estimate of error based on 47 degrees of freedom. It is worth noting that the use of half plant totals by Samuel and Bald (6) corresponds to a group of 7 degrees of freedom which constitute a subsection of the 47 degrees of freedom available for computing the error. Naturally an estimate of the error based on so few degrees of freedom discards a considerable portion of the information available and will not be as dependable an estimate of the error.

The interpretation of these data is made by means of the values listed in the Mean Square column under the Analysis of Variance. These values, together with their attendant Degrees of Freedom, provide a concise summary of the information in the original data. The data may be interpreted in the usual way by using the standard deviation. The square root of the variance (21.14) is the standard deviation of the number of lesions on a single half leaf. There are 48 half leaves receiving each treatment. The standard deviation of the total is $4.598\sqrt{48}$ or 31.85. The standard deviation of the difference between the two treatment totals is $31.85\sqrt{2}$ or 45.05. The observed difference between the totals is 501 minus 394 or 107, or 2.38 times its standard deviation. The same data evaluated by using totals of the six half leaves on each plant show an average difference between the half plant totals of 13.375 ± 7.40 . By this method the difference is less than twice its standard deviation. Information has been lost by forming aggregates of the leaf counts. A glance at the value 119.3 listed opposite Treatments shows this is greater than the value 21.14 opposite Error. One-half the difference of the natural logarithms of these two quantities is compared with the value listed in Fisher's (2) double entry table of z . As previously explained the degrees of freedom of the two mean squares determine the point of entry in the table. The calculated value (0.8653) of z exceeds the quantity 0.7141 given in the five per cent table and it is concluded that the treatments were different. (Allowance should be made for the fact that the 46 degrees of freedom are larger than the 30 which is the nearest given in the table. By interpolation the more accurate value 0.7015 is obtained.)

The totals for the right and left halves may be submitted to the same procedure. The totals differ by 9 with a standard deviation as before of 45.05. The difference is less than its standard deviation and is not significant. The use of the z table confirms this result. The z table does show that the mean squares obtained for plants and for leaf positions differ from the value obtained for error. This furnishes the evidence for stating that individual plants and different leaf positions differ in susceptibility. In these two cases the use of the standard deviation as a criterion is decidedly awkward.

In the column headed Standard Deviation there are listed three values. The best estimate is listed opposite the entry Error and is based on 46 degrees of freedom. It has already been pointed out that the estimate based on the 35 degrees of freedom is the best estimate of the error in the case of experiments with several treatments applied in the design of a Latin square. Evidence will appear in a subsequent table to show that these two estimates are in close agreement. The value 6.04 for the standard deviation is based on 7 degrees of freedom. If this quantity is multiplied by $\sqrt{6}$, and also by $\sqrt{2}$ and then divided by $\sqrt{8}$ the value 7.40 is obtained

which is identical with that found by the use of Student's method applied to the half plant totals. The presence of 6 half leaves in a half plant total accounts for the factor $\sqrt{6}$. The factors $\sqrt{2}$ and $\sqrt{8}$ are introduced because the result applies to the mean *difference* between 8 pairs of half plant totals.

The analysis of variance makes available all the information that may be obtained piecemeal by the application of special methods. Its use should remove the confusion that often arises in the mind of the experimenter as to the proper choice of a statistical method for interpreting data.

TABULATION OF THE RESULTS OF COMPUTATIONS

Since the mean square values and their degrees of freedom suffice to interpret the data, these two columns have been listed for 39 experiments in Tables III and IV. Table III consists of experiments with the half leaf method. The asterisks mark the mean square values that differ significantly from the experimental error. In only two cases do the half leaves contribute to the variance of the observed counts.

The experiments shown in Table IV were made using Holmes' technique. Thus, five leaves on each of 16 plants were inoculated with preparation A. From the 80 leaf counts the experimental error has been computed for an individual leaf. The advantage of computing the experimental error in this way is shown in Figure 2. This figure shows the relation between the probable error of a single plant and the total number of lesions on the plant. Curve A is taken from Figure 10 in Holmes' first article (5) on this method. The squares are the points shown in his figure. The circles are additional values obtained in this work. The data for these points are available in Tables III and IV. The mean square value listed under plants in these tables is simply the sum of the squares of the differences of the individual plant totals from the average plant divided by one less than the number of plants and also divided by 5 in the case of Table IV and by 12 in the case of Table III. These last factors arise through the use of the leaf and the half leaf as the unit in the calculations. Therefore, the probable error of a single plant is found as in following examples:

Table III	Table IV
$0.6745\sqrt{12 \times 36.58} = 14.1;$	$0.6745\sqrt{5 \times 1333.23} = 55.1$

These values plotted against the appropriate average plant totals give the circles for curve A. The circles confirm Holmes' estimate of the probable error of a single plant using plant totals. Curve B shows the reduction in the estimated probable error when the leaf or half leaf units are used

TABLE III
ANALYSIS OF VARIANCE FOR EXPERIMENTS WITH THE HALF LEAF METHOD; EACH VIRUS PREPARATION
WAS PAIRED WITH A FOUR-UNIT PREPARATION

Item	Virus Prep. Units	Treat- ment	L. Half vs. R. Half	Whole Leaves	Error (Vari- ance)	Plants	Leaf Positions	Inter- action	Interac- tion Half Plant Totals	Av. No. Lesions per Plt.	Total Diff. †	Right Minus Left ††
Deg. Free. Mean Sq.	1	I 1533.68**	I 1.01	59 17.08	58	9 36.58**	5 28.74*	45 11.89	9 15.92	69.9	429	-11
"	2	78.41 6.53	9.08 16.13	111.35 20.38	22.31 13.30	308.12** 47.83**	74.31* 86.95**	76.11 19.29	60.11 17.98	92.9	97	33
"	4	1032.53	17.63	93.57	23.26	291.41**	78.24**	55.71	52.57	95.0	-28	44
"	8									144.6	-352	46
Deg. Free. Mean Sq.	1	I 2400.00**	I 84.38	47 65.60	46	7 217.98**	5 161.07**	35 21.49	7 74.07	98.0	480	90
"	2	102.09	46.76	281.36	61.56	556.52**	1084.42**	111.00	32.55	194.6	99	-67
"	4	31.51	58.59	325.06	54.97	581.99**	1017.41**	174.94	36.18	223.6	-55	75
"	8	1776.76**	114.84	320.99	115.72	694.46**	615.46**	204.23	379.12	248.1	-413	105
Deg. Free. Mean Sq.	1	I 765.01**	I 4.59	47 27.55	46	7 66.64**	5 87.24**	35 11.21	7 21.06	57.5	271	21
"	1	270.01**	1.26	14.23	7.85	29.74**	47.61**	6.36	12.46	49.6	161	11
"	2	341.26**	0.09	62.36	11.22	122.27**	275.56**	19.92	12.45	84.9	181	3
"	3	119.26*	0.84	81.50	21.14	117.32**	326.06**	39.40	36.47	111.9	107	-9
"	4	108.38*	32.67	80.89	18.06	162.66**	311.12**	31.65	41.33	115.8	102	-56
Deg. Free. Mean Sq.	1	I 0.88	I 3.80	95 18.83	94	15 30.90**	5 132.18**	75 8.85	15 4.22	66.19	13	27
"	4	42.19	3.52	37.28	5.82	48.30**	387.02**	11.77	14.71	78.88	-90	26
"	4	6.38	115.03**	23.25	8.25	26.00**	171.61**	12.09	7.13	68.44	35	149
"	4	2.30	100.63*	56.18	18.36	139.77	112.36**	35.71	8.19	150.81	-11	139
Deg. Free. Mean Sq.		† ††	I 3340.84** 109.00	49 2058.69 1490.92	49 271.39 144.06	9 4390.58** 2411.34**	4 6024.44** 3863.34**	36 1035.09 997.22	9 236.02 159.27	533.6 347.4	—	-578 -130

* See Table II, footnote *; ** See Table II, footnote **.

† Total number of lesions with four-unit preparation minus total lesions with preparation tested

†† Total number of lesions on right halves minus total number of lesions on left halves.

†† Samuel and Bald (6) Table II; †† Samuel and Bald (6) Table IV.

TABLE IV
ANALYSIS OF VARIANCE OF EXPERIMENTS WITH HOLMES' METHOD

Remarks		Virus Prepa- ration	Plants	Leaves	Error (Variance)	Av. No. Lesions per Plant
Inoculated Jan. 21, 1933	Deg. Free.		15	4	60	
	Mean Square	A	1333.23**	885.83*	261.91	273.6
	Mean Square	B	1154.29	3647.27**	484.75	364.6
	Deg. Free.		24	4	96	
	Mean Square	C	3629.97**	2724.85**	561.71	398.4
	Mean Square	D	1986.93**	2615.67**	253.70	209.8
	Deg. Free.		35	4	140	
	Mean Square	E	2793.96**	4134.18**	318.71	348.6
	Mean Square	F	1074.26**	216.51**	76.69	106.4
	Deg. Free.		7	4	28	
Large plants	Mean Square	G	575.23**	348.65**	61.42	110.1
Large plants de- capitated	Mean Square	G	283.49*	40.29	102.39	106.5
Small plants	Mean Square	G	417.36**	131.16	56.99	62.3
Small plants de- capitated	Mean Square	G	164.00**	20.60	24.36	46.0
Medium plants	Deg. Free.		19	4	76	
Medium plants de- capitated	Mean Square	I	684.10**	263.35*	92.54	136.8
	Mean Square	I	941.50**	305.77	166.62	132.2
	Deg. Free.		8	4	32	
	Mean Square	104	757.44*	1333.75**	313.74	191.8
	Mean Square	I	1231.50**	317.70	295.63	158.0
	Mean Square	102	239.20**	874.95**	62.39	148.3
	Deg. Free.		15	4	60	
	Mean Square	11	321.86**	349.51*	107.19	142.9
	Mean Square	102	318.61	710.11**	183.81	147.4
	Mean Square	G	784.45**	403.33*	124.38	139.0
	Mean Square	F	177.69**	208.28*	73.56	98.8
	Mean Square	103	528.85*	1026.12**	226.71	193.1

* Differs from value in Error column with odds of at least 19 to 1.

** Differs from value in Error column with odds of at least 99 to 1. See Fisher's (2) table of z .

in the computations and the extraneous factors eliminated. The mean square values listed under Error are used to establish the solid dots.

Table III

$$0.6745\sqrt{12 \times 11.49} = 7.9;$$

Table IV

$$0.6745\sqrt{5 \times 261.91} = 24.4$$

These calculations have been made solely to bring the figures to a suitable basis for comparison with the early standard of accuracy. The curves show that the experimental error is really about one-third of the original estimate. This reduces to one-ninth the number of plants required to establish a given difference.

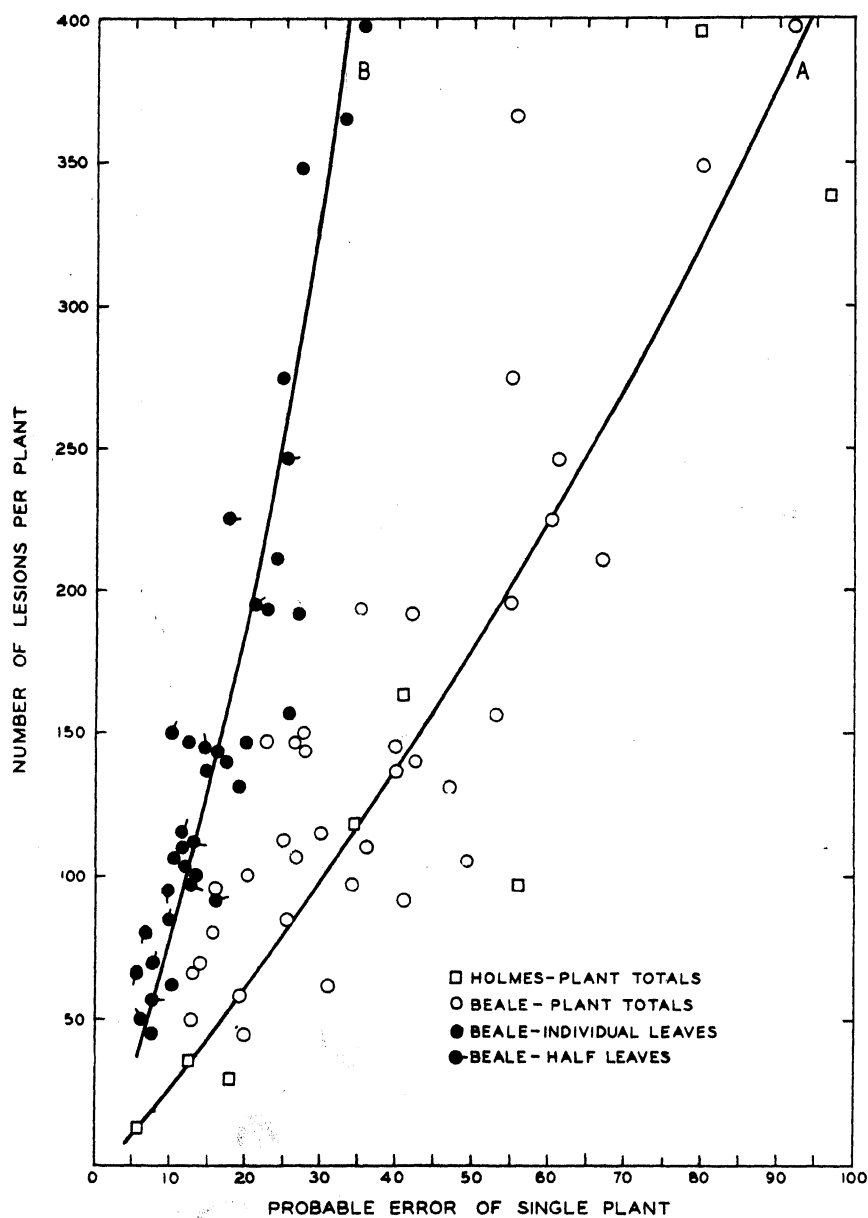


FIGURE 2. Curve A shows the probable error of the number of lesions on a single plant when computed from the plant totals. Curve B shows the experimental error of lesion counts made by the half leaf method or by a method that eliminates the contribution of plant variation and leaf position to the error.

DISCUSSION AND SUGGESTED PROCEDURE FOR
CONDUCTING EXPERIMENTS

Certain of the statements made earlier in the paper may now be reconsidered. One of the first factors mentioned had to do with differences in susceptibility of individual plants. The analysis of variance shows that in all of the 39 experiments the mean square due to this element exceeds that listed for the experimental error. In but two cases are significant odds lacking. Most of the improvement found by Samuel and Bald in comparing two preparations is due to the elimination of the contribution arising from plant differences.

The second source of variation suggested is that due to the position of the leaf on the plant. In 34 of the experiments satisfactory odds exist in support of this contention. One explanation probably accounts for the other five cases. In Figure 1 it appears that the gradient of susceptibility may take several forms. Undoubtedly the gradient instead of being from the top down or the reverse may sometimes be neither. In such cases, of course, the effect of leaf position vanishes. In any event the effect should be considered since in general the elimination of this one source of variation will increase the precision.

Compared with the two considerations just mentioned the possible difference between the two halves of a leaf is not at all important and is not of any consequence in these experiments. This very point will serve to illustrate a valuable feature of this statistical analysis. During the course of work in this field much data must accumulate. The analysis of variance brings about a great condensation in the bulk of the data and makes possible a rapid survey of the whole work. Certain small effects that otherwise would escape notice may finally be established with just as much certainty as is commonly placed in large effects which may, simply because they happen to be large, rest upon relatively few observations.

The details of the calculations made on the possible effect of different pieces of cheesecloth are not included in this paper. The suggestion is made that at least two pieces of cloth be used in applying any given inoculation. If proper records are kept data will eventually be at hand to settle this point and the analysis may then be adapted to allow for the effect.

About half the experiments considered were based upon the half leaf method. This method, of those considered in this paper, is the most accurate for comparing two virus preparations. The most important limitation of the half leaf method is the needless repetition of a reference treatment if more than two are being compared. If six preparations, differing in concentration, are at hand 15 different pairs must be formed if all possible comparisons are desired, as might well be the case if little were known about the solutions. If the data are submitted to the analysis of variance, all this would be avoided by simply inoculating one leaf from each plant with each preparation and rotating the order.

Suppose, for example, 25 plants are available to establish a comparison of five different treatments, A, B, C, D, and E. The arrangement of the applications on the leaves, supposing each column to be a plant, will be as follows:

A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A
C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B
D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C
E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D

In Table IV preparation D is shown as having been tested on 25 plants. Suppose five hypothetical virus preparations (A, B, C, D, and E) are assigned to the leaves according to the above scheme, and the leaf counts totalled for the 25 A leaves, the 25 B leaves, and so on. This was actually done and the five totals, 1051, 1069, 1078, 1053, and 995, were obtained. The totals should be the same since the five preparations are really the same. In other words, this is a method of dividing the 25 plants into five really comparable samples upon which five different treatments might have been tested. These same plants were divided at random into five sets of five plants each. The totals were then 849, 1285, 1065, 1364, and 685. This illustrates the differences in susceptibility encountered in groups of plants selected from the same stock. These are not so uniform as the samples actually available from these plants if selected as first described. This uniformity is not unusual. Plants tested with preparation E (Table IV) were divided into five samples, first by the suggested method and then into random sets of seven plants. In the first case the five samples showed lesion counts of 2534, 2512, 2433, 2438, and 2385 which contrast greatly with the totals of 3067, 2488, 2217, 2065, and 2465 if the samples consist of five sets of seven plants each.

Another merit is attached to this experimental arrangement. Even though extracts differing in concentration are applied to the plants, the same treatments occur equally often at each leaf position and on each plant. The plant totals and the leaf position totals, therefore, are still available as indicators of the uniformity of the plants. So that while actually conducting experiments on the virus, the uniformity of the plants at different times of the year may be accurately compared through the analysis of variance. Or the uniformity of different arrangements for growing the plants, or different stocks of seeds, may be compared without in any way interfering with the use of the plants for comparing virus preparations.

It may happen, in certain experiments conducted on a whole leaf basis as suggested, that some of the preparations vary greatly in concentration. For example, it might be that two out of five preparations are much

stronger than the other three. The analysis of variance gives one experimental error to apply to the whole set. This is obviously an injustice since Figure 2 shows that the error is a function of the number of lesions. It is not difficult to circumvent this. The data for the two strong solutions are simply segregated from the rest. For each set, plant totals, leaf position totals, and treatment totals must be formed and the analysis made separately for each set, using its own totals. If the treatment totals are the first ones summed it will permit a decision to be made before needlessly forming any others.

If the inoculums do not differ greatly in concentration (and they should be subdivided after the experiment so that this is true) the use of a generalized experimental error is quite justified. If they are of nearly equal strength, the counts for each will also be but little different. The use of a joint error will be more nearly correct than efforts to establish a particular error for each treatment.

The Latin square has an advantage over the half leaf procedure for comparing several extracts or dilutions. In the latter case the data are interpreted by means of differences. Even if only three extracts, A, B, and C, are involved and two experiments arranged for the pairs AB and AC, difficulties are encountered immediately. It is presumed that B and C may be compared through the medium of A. But A is not the same in both pairs. Two different sets of plants were used for the A paired with B and for the A paired with C. This brings the plant variation back into the problem unless an experiment is also performed with the pair BC. Even then the net result is the knowledge of A minus B, A minus C, and B minus C, all on different sets of plants. The Latin square method will give definite counts that may be assigned to the several treatments and which may be freely intercompared since each appeared on every plant and in every leaf position. However, if two sets of extracts are tested at different dates, plant variation is still a limiting factor.

SUMMARY

A large amount of data obtained in measuring tobacco mosaic virus has been submitted to statistical reduction, employing the analysis of variance. By this means the degree of variability of different plants is accurately estimated and the existence of a gradient of susceptibility between the different leaf positions established. The nature of this gradient varies with different sets of plants. It was shown that the right and left halves of a leaf respond equally to the inoculation procedure as employed in this laboratory.

The analysis of variance has been applied to the data in order to show the actual experimental error of the lesion count obtained from a single leaf. This error is approximately one-third of the error reported in the

original description of this method and this reduces the number of plants required to establish a given difference between virus preparations to about one-ninth, or trebles the accuracy if the same number of plants is retained. An experimental arrangement has been designed to permit the intercomparison of a number of virus preparations without unnecessary duplication of a reference standard. This is accomplished by so distributing the several treatments among the leaves that each appears equally often on each plant and at each leaf position.

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TOXICITY OF AIR CONTAINING SULPHUR DIOXIDE GAS

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The dangers involved in attempting to grow plants in or near industrial centers have been discussed from early times in numerous publications. The first major interest in the United States arose in connection with operating a smelting plant in California in 1905. The people of the city of Benicia and its surrounding country brought an action in court asking for an injunction which would restrain the Selby Smelting & Lead Company from permitting the smoke of the Selby Smelter to blow over their territory. After more than three years of litigation, judgment was rendered in favor of the plaintiffs. The smoke nuisance, however, to a lesser degree, continued until, in 1913, the Selby Smelter Commission was appointed to make a thorough study of conditions and recommend possible solutions to the problems involved. Experts were employed and a thorough study was made of toxic ingredients in dust, fumes, gas, and smoke from the stacks of the metallurgical plants. The Commission determined that practically all of the injury to vegetation was due to sulphur dioxide gas which was liberated in large amounts and was carried for long distances in sufficient concentration in the atmosphere to cause injury to plants. A published report (2) of the Commission appeared in 1915. Included in the report is an extensive list of references to and abstracts of literature available up to that time. For that reason, no further review of the early work will be made in the present paper. In 1923, Stoklasa (7) published the results of his investigations on the toxicity of smoke gasses to vegetation, and made many references to literature. In 1932, Haselhoff, Bredemann, and Haselhoff (1) published an extensive review of the known literature on the subject of smoke injury. All three of these publications are valuable for those interested in the toxicity of smoke.

As might be expected, the published results are not all in agreement concerning the minimum concentrations causing injury or conditions under which plants are most susceptible. Due to this need for additional information, many experiments were conducted under controlled conditions at the Boyce Thompson Institute. The present paper reports the results obtained when plants were exposed to known concentrations of sulphur dioxide gas under a number of different conditions for various time periods during the summer months of 1930, 1931, and 1932.

METHODS AND MATERIALS

Fumigation chambers were made from window sash fastened together so as to make glass cases seven feet long, three feet high, and three feet wide. The middle of one side was fitted with a door which could be easily

opened when plants were to be inserted or removed. One end was fitted with an adjustable exit and the other an inlet for admitting the sulphur dioxide air mixture. A motor-driven blower forced the air through a meter and then through the pipe leading into the glass case. The gas, coming from a small tank of liquid sulphur dioxide, was admitted to the air in the pipe between the meter and the case, allowing a distance of six feet before the mixture reached the experimental plants. When the blower was going at full force, 15 cubic feet of air per minute passed into the fumigation chamber. In admitting the sulphur dioxide, the rate was governed by means of a venturi meter (flow meter) made of a U-tube, two T-tubes, and a capillary tube. The length and diameter of the capillary tube determined the pressure necessary to deliver a given quantity of the gas per unit of time. Several capillaries were calibrated to fulfill the needs under different conditions of the experiments. After careful calibration, the tubes could be relied upon with constant pressure to deliver a desired quantity of gas within a range of five to ten per cent. Mercury or paraffin oil was used in the U-tube. Water was not used because it is a solvent for sulphur dioxide and the moisture clogged the fine capillary tube. The height of the column of mercury in the arm of the U-tube was not relied upon as a final indication of the quantity of sulphur dioxide being delivered even after the capillary tubes were calibrated, but as final proof analyses were made of samples of air withdrawn from amongst the plants in the case. The apparatus and methods for analysis were essentially as described by the Selby Smelter Commission (2, p. 200 ff.). Several modifications were made to meet local conditions. For example, since there was not a measurable amount of sulphur dioxide at any time in the atmosphere in the vicinity where the experiments were conducted, it was not necessary to use an air-tight system between the sample bottles (aspirators) and the test bottles, the starch solution being poured directly from one to the other.

The starch solution was prepared by adding one gram of soluble starch to one liter of boiling water. After cooling this was brought to a pronounced blue with a standard iodine solution and kept in a cool place until used. Some difficulty was experienced in obtaining starch which gave a good blue color, instead of purple, when iodine was added. Two grams of potassium iodide per liter of starch solution seemed to intensify the blue color.

The steps in the procedure for making a determination were as follows:

1. Two hundred fifty cc. of the standard blue starch solution (made up shortly before needed) were poured into each of two 12-liter aspirators fitted with glass tubing through two-hole stoppers for attachment of manometer and vacuum pump.
2. The aspirators were evacuated to a residual pressure of 260 to 360 mm. and carried to the fumigation chamber where one was again connected

with a manometer and after a reading was made the sample was taken by opening a stopcock attached to a glass tube leading into the case where the experimental plants were located. The stopcock of the control aspirator was opened anywhere in the vicinity of the fumigation chamber. With this method, the pressure in the aspirators was brought back to normal.

3. The aspirators were shaken 50 to 60 times in such a way that the walls were repeatedly wetted with the starch solution.
4. The aspirators were taken back into the laboratory and the starch solutions transferred to wide-mouthed, 400-cc. bottles. At this time, there could be seen a difference in color of the two solutions as a result of the presence of sulphur dioxide in the treated air.
5. By means of a micro-burette $n/1000$ iodine solution was added, drop by drop, to bring the color in the "test" bottle back to that of the "blank." The number of cubic centimeters of iodine used indicated the amount of sulphur dioxide absorbed.
6. An actual example is as follows:
 - a. 1 cc. $n/1000$ iodine solution is equivalent to 0.000032 gram of sulphur dioxide, equal to 0.0112 cc. sulphur dioxide at 0° C. and 760 mm.
 - b. Temperature of air, 27° C.
 - c. Barometric pressure, 760 mm.
 - d. Capacity of aspirator, 12708 cc.
 - e. Extent of evacuation, 582 mm.
 - f. cc. of $n/1000$ iodine required in titration, 0.78.

Calculation from above data:

$$(1) \text{ 0.78 cc. of } n/1000 \text{ iodine} = 0.78 \times 0.0112 = 0.00873 \text{ cc. of sulphur dioxide at } 0^{\circ} \text{ C. and 760 mm.}$$

$$(2) \text{ Volume } 12708 \text{ cc.} \times \frac{582}{760} \times \frac{273}{300} = 8860 \text{ cc.}$$

$$(3) \frac{0.00873}{8860} = 0.000098 \text{ per cent or 0.98 part of sulphur dioxide per million of air.}$$

Plants used in the experiments were grown in pots either in green-houses or in frames, depending upon the nature of the experiments. Where comparisons of susceptibility were to be made, plants were grown in composted soil under uniform conditions. The entire list of plants is as follows: alfalfa (*Medicago sativa* L.), barley (*Hordeum vulgare* L.), buckwheat (*Fagopyrum esculentum* Moench), castor bean (*Ricinus communis* L.), Chinese cabbage (*Brassica chinensis* L.), coleus (*Coleus blumei* Benth.), cos lettuce (*Lactuca sativa* L. var. *longifolia* Lam.), egg plant (*Solanum*

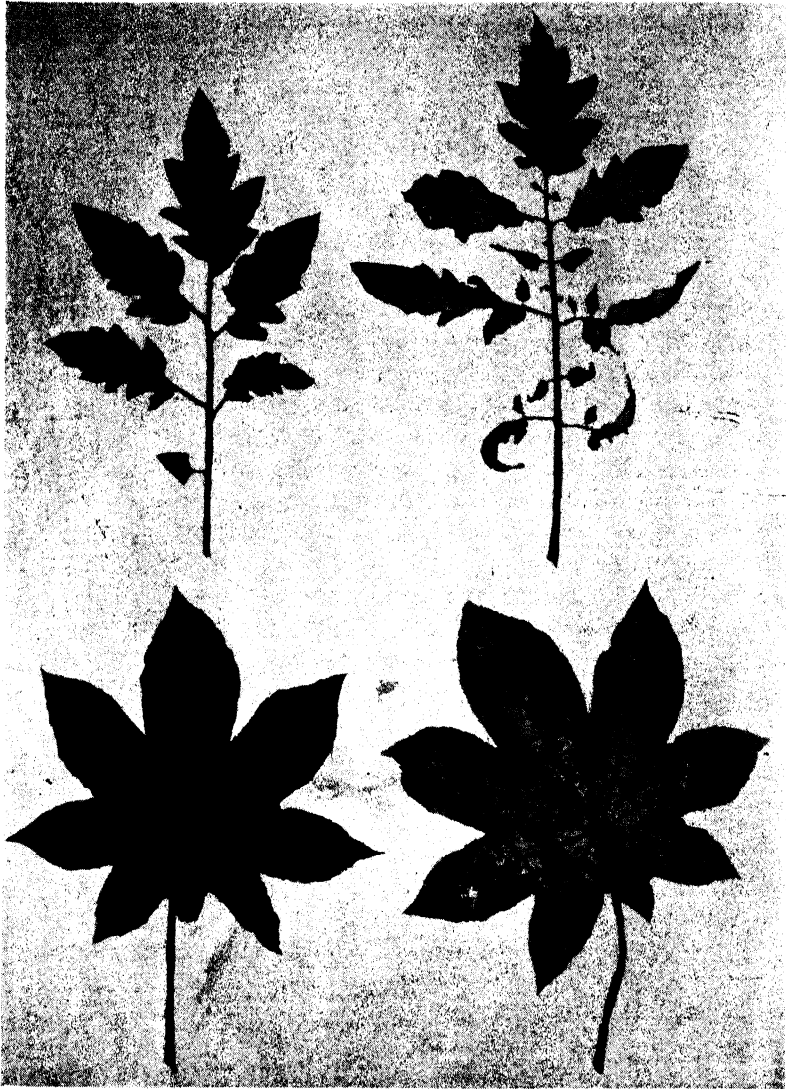


FIGURE 1. *Above.* Tomato leaves. Left, control leaf; right, leaf treated with 4 parts of SO_2 per million of air for three hours. *Below.* Castor bean leaves. Left, control leaf; right, leaf treated with 8 p.p.m. for three hours.

melongena L.), French endive (*Cichorium endivia* L.), lily (*Lilium speciosum* Thunb. var. *Rubrum*), meadow fescue grass (*Festuca elatior* L.), New Zealand spinach (*Tetragonia expansa* Murr.), oats (*Avena sativa* L.), orchids (*Cymbidium* sp., *Oncidium* sp., *Cattleya* [3 species], and *Odontoglossum* sp.), pepper (*Piper nigrum* L.), radish (*Raphanus sativus* L.), rose (*Rosa* [hybrid tea] var. *Madame Butterfly*), rye (*Secale cereale* L.), salvia (*Salvia splendens* Ker.), sweet clover (*Melilotus alba* Desr.), sweet pea (*Lathyrus odoratus* L.), Swiss chard (*Beta vulgaris* L. var. *cicla* L.), timothy (*Phleum pratense* L.), tomato (*Lycopersicon esculentum* Mill.), turnip (*Brassica rapa* L.), wheat (*Triticum aestivum* L.).

EXPERIMENTAL RESULTS

During the course of these investigations, especial attention was paid to the following responses and factors:

1. Characteristic injury caused by sulphur dioxide gas.
2. Extent of injury with various concentrations and fumigation periods.
3. Internal and external factors as affecting susceptibility.
4. Susceptibility of species to sulphur dioxide fumigation.
5. Injuries caused by fumes from a smelter plant.

1. *Characteristic injury caused by sulphur dioxide gas.* Plants which were fumigated with known concentrations of sulphur dioxide gas showed certain injuries which were characteristic for the species and for the age of the tissues involved. Leaves of dicotyledonous plants became spotted and marked between the veins. The tissue about the veins was comparatively resistant, frequently showing no injury, while much of the interveinous tissue was killed (Fig. 1). Leaves were never uniformly attacked unless the concentration of sulphur dioxide in the air was very high and the plants were fumigated for long periods. With low dosages, dead patches appeared at various places on the leaves without relation to the age of any part of that particular organ. The age factor was important, however, on plants as a whole, if they held young to old leaves at the time they were fumigated. Annual plants growing in June, July, or August were, as a rule, of this sort. In such cases, the middle-aged leaves were the most sensitive, followed by the old and then the growing leaves, which were the most resistant. These results seem to be correlated with the size of the opening of stomata of different aged leaves. Knight (3, p. 75) reported that stomata of a mature healthy leaf ("middle aged") may open more widely than those of a very young or very old leaf. Leaf buds and developing leaves withstood high dosages which killed all of the older leaves on the plants. Upon removal from the sulphur dioxide fumes, most species severely injured started to make recovery from all growing points. Plants treated with mild dosages usually appeared normal for 24 hours or more after the fumigation was stopped. This was particularly striking if the plants were

kept in the shade. Upon being exposed to direct sunlight, injuries appeared as light colored specks or patches on interveinous tissue, and, for some species, along the edge of the leaf blade. The light color of the patches was due to the fact that the tissue at such places was killed, and was then bleached upon drying. If observed while plants were being exposed to high concentrations of the gas, the tissue had the appearance of being cooked by hot water.

The response of monocotyledonous leaves to sulphur dioxide fumigation varied from slight mottling or injured tips with dilute gas to completely bleached leaves treated with high concentrations of gas. Between

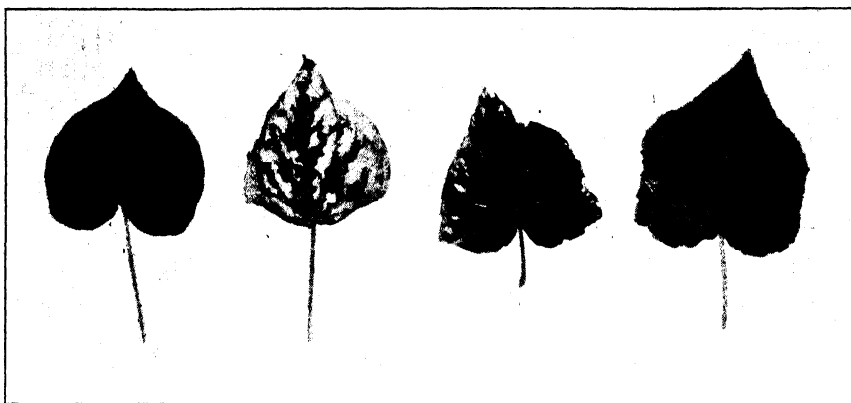


FIGURE 2. Buckwheat leaves from plants treated for six hours with various concentrations of sulphur dioxide gas. Left to right, (1) control, (2) 1.0 p.p.m., (3) 0.7 p.p.m., (4) 0.5 p.p.m.

these two extremes could be found leaves with various degrees of mottling from injured patches. Leaves of young plants were attacked first at the tip, the injury extending downward with increased concentrations and length of fumigation periods. Injury on monocotyledonous types could be distinguished from that of dicotyledonous types by the parallel effect of injured regions and veins on the former.

2. *Extent of injury with various concentrations and fumigation periods.* All other factors being equal, the amount of injury varied with the concentration of the gas and the length of the fumigation period. Many irregularities appeared when the plants used were lacking in uniformity as to age, activity, and conditions under which they were grown. In order to get uniform plants for the experiments, in some cases seeds were sown in six-inch pots and the resulting seedlings thinned to a uniform number of plants per pot. At the time they were fumigated, buckwheat, for example, had six plants per pot with 8 to 10 leaves each, or 48 to 60 leaves

per pot. At the beginning of each experiment, enough pots of plants were enclosed in the glass case so that one or more could be removed each hour for the duration of the fumigation. Check plants were kept in a nearby glass cage away from the sulphur dioxide fumes. The temperature in the case varied with that of the surrounding air, and when the sun shone, though the cases were shaded with cloth, the temperature inside exceeded that outside by several degrees. Upon removal from the cases, the plants were placed in light to be left 24 to 48 hours before the final readings were made. As a rule, the damage could not be easily seen until after the injured patches had been bleached in direct sunlight.

Buckwheat was the most susceptible species discovered in the course of these investigations. With continuous exposure to the gas, leaves were slightly injured in seven hours with 0.46 of one part of sulphur dioxide per million of air. Approximately half of the leaves were injured within an hour when the plants were exposed to 0.80 p.p.m. In general, it appeared that the injury to buckwheat leaves increased directly with the concentration of the gas and the length of the fumigation period (Fig. 2, Table I). The same trend was shown by fumigated barley, rye, and oats, though they were not so susceptible as buckwheat. Barley was slightly injured in eight hours when exposed to 0.60 p.p.m.; in four hours with 0.66 p.p.m.; and in two hours with 0.80 p.p.m. Oats and rye treated in

TABLE I
INJURY TO BUCKWHEAT PLANTS FUMIGATED WITH DIFFERENT CONCENTRATIONS
OF SO₂ FOR VARIOUS TIME PERIODS

Conc. of SO ₂ expressed in parts per million	Amount of injury* with various hours of fumigation						
	1	2	3	4	5	6	7
0.46	o	o	o	o	o	o	x
0.54	o	o	o	o	o	x	
0.60	o	o	x	xx	xxx	xxx	xxx
0.66	x	xx	xxx	xxxx			
0.80	xx	xxx	xxx	xxxx			

* o=no visible injury; x=slight injury on few leaves; xx=approximately half of the number of leaves injured; xxx=approximately three-fourths of leaves injured; xxxx=practically all leaves marked.

the same case with the barley showed approximately the same amount of injury. In most cases, the number of leaves marked with injury increased irregularly with the length of the fumigation period as shown in Table II. The irregularity is assumed to be due to differences in the internal condition of individual plants as will be shown later.

3. *Internal and external factors as affecting susceptibility.* One of the greatest difficulties encountered in conducting experiments to determine the toxicity of sulphur dioxide was to obtain uniform plant material. Plants

TABLE II
INJURY TO BARLEY, RYE, AND OATS FROM FUMIGATION WITH 0.80 P.P.M. OF SO₂

Plants	Number of leaves injured during different fumigation periods									
	2 hr.		3 hr.		4 hr.		5 hr.		6 hr.	
	Total No. of leaves		Total No. of leaves		Total No. of leaves		Total No. of leaves		Total No. of leaves	
	On plants	In-jured	On plants	In-jured	On plants	In-jured	On plants	In-jured	On plants	In-jured
Barley	20	3	15	5	16	3	14	6	29	9
Rye	20	4	29	19	25	25	28	20	35	33
Oats	26	2	25	6	25	2	35	3	36	11

of uniform size and age grown in one pot did not always show uniform responses when fumigated with sulphur dioxide gas. Opposite leaves of coleus plants were not always equally sensitive to the fumes. Of approximately one thousand leaflets on alfalfa plants in one pot, only a few, possibly 3 to 10, might show injury, whereas there were many of the same age, size, and appearance. These facts indicate that the internal conditions at the time the plants are fumigated may control the entrance of sulphur dioxide and thereby affect the resistance of the plants.

Of the many possible factors which might play a part in causing resistance to sulphur dioxide, it appeared that the degree of turgidity (8) of leaves at the time they were fumigated was one of the most effective. For

TABLE III
COMPARATIVE INJURY OF SO₂ FUMES ON PLANTS THAT WERE WILTED VS. TURGID

Plants	Conc. of SO ₂	Condition of the plants	Extent of injury* with different fumigation periods					
			1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.
Buck-wheat	0.98	Turgid	x	x	xx	xxx	xxx	xxxx
	0.98	Wilted	o	o	o	o	o	x
	1.44	Wilted	o	o	o	o	o	
Tomato	1.13	Turgid						xx
	1.13	Wilted						o
	4.2	Turgid	xxx	xxx	xxx		xxxx	
	4.2	Wilted	o	o	o		o	
Sweet clover	0.82	Turgid	o	o	x	xx		
	1.44	Wilted	o	o	o	o	o	o
Salvia	4.0	Turgid	xx	xxxx	xxxx			
	4.0	Wilted	o	o	x			
Coleus	4.0	Turgid	xx	xxx	xxxx			
	4.0	Wilted	o	o	o			

* o = no injury; x = slight injury; xx = moderate injury; xxx = considerable injury; xxxx = all leaves injured.

experimental purposes, several species in pots were allowed to dry to the point where wilting was evident and then exposed together with turgid plants to sulphur dioxide gas. The results, shown in Table III, indicate that wilted plants were much more resistant than turgid ones. For example, with 0.98 p.p.m. turgid buckwheat plants were slightly injured within an hour and all of the leaves injured in six hours, whereas wilted plants were only slightly injured with the same concentration in six hours. Turgid tomato plants were severely injured when fumigated with 4.2 p.p.m. for five hours contrasted with no injury on wilted plants given the same treatment. Similar results were obtained with clover, alfalfa, salvia, and coleus (Fig. 3). Neger and Lakon (5) observed that a conifer branch which had been partially severed and was therefore slightly dry was more resistant to sulphur dioxide than the other branches which were fresh. Sayre (6) observed that stomata of healthy leaves of *Rumex patientia* were always closed at night. They were always open in the daytime if the leaves were not wilted. On wilted leaves the stomata were found closed or nearly closed. Loftfield (4) found that stomatal opening and closing was closely correlated with light and dark when the leaves were turgid, but with a water loss sufficient to cause a saturation deficit, stomata closed. In our experiments badly wilted plants were more resistant than those just beginning to wilt. The variation in susceptibility among many plants in one pot or many leaves of the same age on one plant may have been due to different degrees of turgidity in otherwise comparable parts. Since stomata are known to start closing when the moisture in the cells approaches a certain saturation deficit, and to close completely when plants are definitely wilted, it seems logical to assume that wilted plants were resistant to sulphur dioxide because, with the epidermal pores closed, the gas could not enter the tissue. This assumption is supported also by the fact that plants are more resistant to sulphur dioxide at night when stomata were closed, or at least partially so, than while in daylight. Buckwheat, tomato, alfalfa, sweet clover, oats, rye, and barley fumigated with 0.85 p.p.m. for four hours between 8:00 p.m. and 12:00 p.m. showed no sign of injury. Comparable plants treated with 0.80 p.p.m. during daylight showed considerable injury with one to two hours of exposure to the gas.

Shading of the plants while being fumigated was not effective in preventing injury from sulphur dioxide, but plants that were kept in the dark for two hours prior to fumigating in a dark case were more resistant than comparable plants treated while in light. The effect of light and dark as factors causing a difference in resistance of plants to sulphur dioxide gas is shown in Table IV. The increased resistance of the plants in the dark may have been due to partial closing of the stomata. They were not immune from attack, but gained some protection from being placed in the dark prior to and during the fumigation period. Other experiments per-

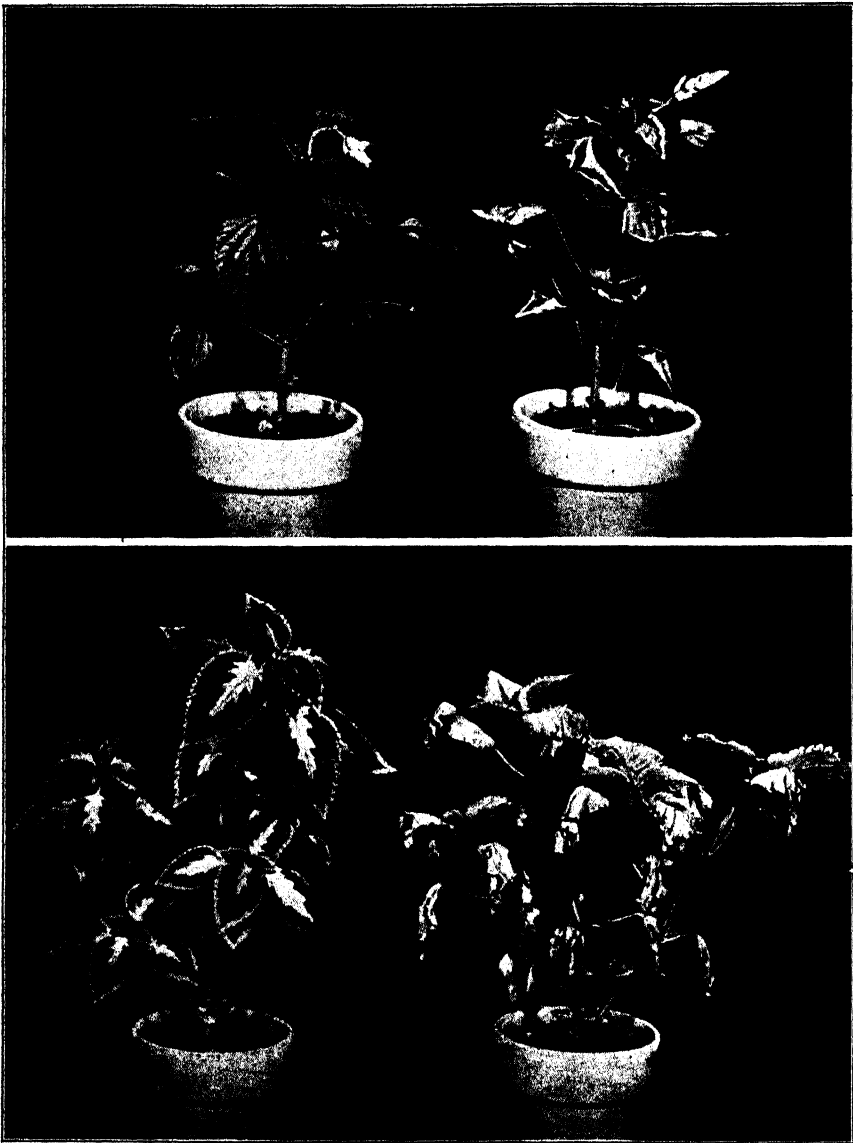


FIGURE 3. Plants fumigated with 4 parts of SO_2 per million of air for three hours while together in a glass case. *Above*. Salvia plants. Left, plant which was wilted prior to and during treatment; right, plant in turgid condition while being treated. *Below*. Coleus plants in the same order and given the same treatment as salvia.

TABLE IV
 SO₂ INJURY ON PLANTS FUMIGATED IN LIGHT VS. DARK*; CONCENTRATION OF THE GAS
 VARIED BETWEEN 1.10 AND 1.17 P.P.M.

Plants	Fumigation		Average temp. ° F.	Average humid- ity	Total No. plants treated	Total No. plants injured	Per- centage injured
	Time in hours	In light or dark					
Wheat	1	Light	74	80	20	4	20.0
	1	Dark	81	75	21	5	23.8
	2	Light	78	76	26	4	15.4
	2	Dark	81	72	27	6	22.2
Barley	1	Light	74	80	24	21	67.5
	1	Dark	81	75	23	12	52.17
	2	Light	78	76	24	24	100.0
	2	Dark	81	72	26	13	50.0
Oats	1	Light	74	80	30	18	60.0
	1	Dark	81	75	32	7	21.87
	2	Light	78	76	29	27	93.1
	2	Dark	81	72	29	14	48.2
Rye	1	Light	71	80	22	13	59.0
	1	Dark	81	75	21	3	14.3
	2	Light	78	76	17	15	88.3
	2	Dark	81	72	17	3	17.64
Buckwheat	1	Light	74	80	20	15	75.0
	1	Dark	81	75	23	14	60.87
	2	Light	78	76	19	18	94.73
	2	Dark	81	72	16	9	56.25

* Plants treated in the dark were held in dark room for 2 hours before treatment began.

formed with plants taken from sunlight directly to the fumigation chamber, indicated that the dark condition is not very effective in bringing about resistance unless the plants have the advantage of a dark period prior to the time they are exposed to the gas. In no case was the darkened fumigation chamber as effective in bringing about resistance as the wilted condition of the plants.

It has been stated that plants fumigated while in high relative humidity are less resistant than in dry atmosphere (2, p. 291). This conclusion was drawn in part from results obtained by comparing injury caused by fumigating a field plot in the morning when the plants were covered with dew as against plants fumigated in the afternoon. From the light of the present experiments, it would seem that the internal condition of the plant is more important than this external factor. In our experiments, 20 per cent difference in relative humidity did not cause any difference in susceptibility of the plants (Table V). There is still a possibility that a wider difference in relative humidity might be responsible for a difference in susceptibility or resistance of plants; however, there are no quantitative data supporting the assumption. Plants growing in low places in fields are said to be more

TABLE V
COMPARATIVE INJURY TO CEREALS FUMIGATED WITH A CONCENTRATION OF 1.35 P.P.M. OF SO_2 WHILE IN DIFFERENT RELATIVE HUMIDITIES

Plants	Hours fumigated	Average temp. °F.	Average humidity	Total No. plants treated	Total No. plants injured	Percentage injured
Wheat	1	85	53	24	1	4.166
	1	84	76	23	0	0
	2	86	51	22	0	0
	2	85	73	32	1	3.12
Barley	1	85	53	36	6	16.66
	1	84	76	33	6	18.2
	2	86	51	38	18	47.36
	2	85	73	36	25	69.44
Oats	1	85	53	36	6	16.66
	1	84	76	35	2	5.7
	2	86	51	32	3	9.37
	2	85	73	30	4	13.33
Rye	1	85	53	34	2	5.88
	1	84	76	30	0	0
	2	86	51	42	5	11.9
	2	85	73	28	1	3.57

susceptible to fumes emanating from smelters than similar species on the upland, the assumption being that the difference is due to high relative humidity in the lowland (2). Loftfield (4, p. 75) found that a high relative humidity of the air permits the stomata to open wider and remain open longer than a low humidity under most conditions. This was especially true where the plants had difficulty in obtaining sufficient water to meet evaporation during the day.

4. *Susceptibility of species to sulphur dioxide fumigation.* As stated earlier in the text of this paper, the extent of injury from fumigation with sulphur dioxide increased with the concentration of the gas and the time the plants were exposed. The minimum dosages which would cause slight injury were not easily determined. Three factors at least are involved—concentration of the gas, the length of the fumigation period, and the internal condition of the plants. The first two are easier to control than the third. This fact is easily demonstrable by showing that a set of 20 similar plants growing in one pot exposed to a given concentration for a given period of time may have only half of the plants injured. In an effort to obtain reliable data, large numbers of plants were used in the experiments to determine effective minimum dosages. The results are shown in Table VI. From these data, it will be seen that under the conditions of our experiments, the species were not equally sensitive to sulphur dioxide gas. In all cases, however, lower dosages for longer periods of time might have been

TABLE VI
MINIMUM DOSAGES OF SULPHUR DIOXIDE GAS WHICH, UNDER THE CONDITIONS OF THESE
EXPERIMENTS, CAUSED INJURY TO VARIOUS SPECIES OF PLANTS

Concentration of SO ₂ in p.p.m.	Species of plants	Hours of fumigation required
0.46	Buckwheat	7
0.60	Barley	8
0.66	Oats Rye Alfalfa Sweet clover Tomato	3 3 5 4 5
0.70	Rose var. Madame Butterfly	6
0.80	New Zealand spinach Meadow fescue grass Timothy Wheat	2 1 1 1
0.825	Sweet pea	3
0.98	Castor bean Salvia Pepper Swiss chard Radish Chinese cabbage	4 5 7 1 2 1
1.05	Turnip Cos lettuce	2 2
1.08	Egg plant Endive, French	1 1

effective. Buckwheat was found to be the most sensitive species, requiring only 0.46 p.p.m. for seven hours to cause injury. Peppers were not injured with less than 0.98 p.p.m. for seven hours. Several species not listed in the table were resistant to high concentrations of sulphur dioxide for several hours. For example, the *Rubrum* lily and several genera of orchids (*Cymbidium*, *Oncidium*, *Cattleya* [3 species], and *Odontoglossum*) resisted 4.3 p.p.m. for five hours with no signs of injury to either leaves or exposed roots. In fact, several orchids resisted 60 p.p.m. for several hours (8). These, however, are exceptions, since the majority of plant species show injury when fumigated with one part of sulphur dioxide per million of air for one to seven hours.

5. *Injuries caused by fumes from a smelter plant.* The authors had occasion to view injured truck crops near a copper smelter during the autumn of 1932. From descriptions of gardeners and appearances of the vegetation, the fumes had drifted with heavy fog for a distance of two miles or more and remained in contact with the vegetation long enough to cause con-



FIGURE 4. Specimens taken from the vicinity of a copper smelter after injury had occurred during heavy fog. Left to right, (1) spinach, (2) beet, (3) sumac.

siderable damage to crops. As seen 24 hours after the attack, the garden plants looked as if they had been fumigated with approximately one part of sulphur dioxide per million of air for an hour or two. The gardeners thought the fog remained over the garden for only 30 minutes. If so, the sulphur dioxide content of the air must have been high to cause so much injury. Figure 4 shows samples of leaves that were taken from the gardens and fence rows of the affected region. By following a wide zone of injured vegetation, it was possible to locate the smelter from which the toxic fumes emanated. Some of the wild plants, especially those of the buckwheat family and the sumacs, were good indicators of where the sulphur dioxide passed. The following plants in the vicinity of the copper smelter were marked with injury:—cultivated plants: *Allium* sp., *Apium graveolens* L., *Beta vulgaris* L., *Cosmos* sp., *Dahlia* sp., *Daucus carota* L., *Endivia* sp., *Lactuca sativa* L., *Lycopersicon esculentum* Mill., *Petroselinum hortense* Hoffm., *Pyrus malus* L., *Rheum rhaponticum* L., *Rubus* sp., *Spinacia oleracea* L., *Taraxacum officinale* Weber., and *Vaccinium* sp.; wild plants: *Acer rubrum* L., *Amaranthus* sp., *Aronia* sp., *Chenopodium album* L., *Galinsoga* sp., *Liquidambar straciflua* L., *Myrica carolinensis* Mill., *Nyssa sylvatica* Marsh., *Polygonum* sp., reed grass, *Rhus glabra* L., *R. toxicodendron* L., and *Sassafras variifolium* (Salisb.) Ktze.

SUMMARY

1. Plants varied in the susceptibility of leaves to injury from sulphur dioxide gas according to the age of the tissues involved. The first leaves to be attacked, if the plants were in an active state of growth, were the middle-aged leaves. The young or growing leaves were the most resistant.
2. Interveinous portions of leaves, though never uniformly attacked, were more readily injured than tissues along the veins.
3. Other factors being equal, the injury increased with the length of the fumigation period and with the concentration of the gas.
4. Wilted plants were more resistant than similar plants that were turgid. The difference was considered to be due, at least in part, to the condition of the stomata.
5. Plants fumigated at night showed more resistance than similar plants treated during the day. Plants also gained in resistance if they were placed in the dark two hours before they were fumigated in a dark case. Shading while plants were being exposed to the gas was not effective.
6. A difference of 20 per cent relative humidity (range between 50 and 75 per cent) did not cause the plants to become more or less resistant.
7. Buckwheat was more susceptible to injury than any other species tested, being marked with injury from fumigation with 0.46 p.p.m. for seven hours. The majority of species were injured when treated with 1.00

p.p.m. for one hour. Orchids were the most resistant of all species, withstanding 60.00 p.p.m. for several hours.

8. A list of plants which were susceptible to sulphur dioxide injury while growing in the vicinity of a copper smelter is given in the text of the paper.

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COTTON FIBERS. IV. FIBER ABNORMALITIES AND DENSITY OF THE FIBER MASS WITHIN THE BOLL

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Microscopic observations of mature fibers of three varieties of cotton, *Gossypium barbadense* L. variety Pima and *G. hirsutum* L. varieties Super Seven and Acala, revealed the presence of few abnormalities in Pima, a moderate number in Super Seven, and a large number in Acala. Similar observations of developing fibers indicated that very few abnormalities could be found in any one of the three varieties until after the twentieth day from the date of flowering.

The bolls had attained their maximum size at about this same time. A rapid increase in size took place during the first thirteen or fourteen days after flowering, a more gradual rate from the fifteenth to the nineteenth days, followed by a cessation of boll enlargement on the twentieth and twenty-first days. Until about the twentieth day, therefore, the increase in size of the boll cavity kept pace, in some measure, with the constantly increasing mass of seeds and fibers within.

During the late stages of boll-development, a sharp contrast was found in the general properties of the bolls in the three varieties. In Pima, the mass of fibers was loosely arranged and the boll comparatively soft. In Super Seven the packing of fibers was more dense and the boll more rigid. In Acala, the very compact arrangement of the fibers and the hardness of the boll suggested that a considerable amount of pressure might have been developed throughout the fiber mass.

An examination of cross sections of cotton bolls such as those shown in (1, Fig. 1) indicated that after the first few days from the date of flowering there is little opportunity for the development of a rapidly elongating fiber in an uninterrupted direction. The seeds themselves occupied a large portion of the available space in the boll cavity. The mass of fibers growing from the surfaces of the seeds conformed, therefore, to the irregular contour of the remaining space. The wave-like or crumpled appearance of the fiber mass just previous to dehiscence is obviously the result of growth within a limited space. Upon the basis of these general observations, it seemed that a causal relationship might also exist between the density of the fiber mass and the number of fiber abnormalities. The problem consisted therefore in obtaining data which would be in the proper form for accurate comparison.

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MATERIAL AND METHODS

The measurements upon which this report is based have been confined to the three varieties, Pima, Super Seven, and Acala. In the course of the study both greenhouse- and field-grown plants were used. Two acres of Super Seven were grown at the South Carolina Experiment Station in 1930.² In this first period of the work attempts to measure the density of the fiber mass within the bolls by means of special mechanical devices were unsuccessful. The method finally chosen consisted in the determination of the weights and volumes of the walls and partitions and of the seeds and fibers separately from bolls at each daily stage of development. The values obtained expressed in a very definite way the relative amount of growth in the separated tissues during the different periods of boll development and indicated indirectly the relative density of the mass of fibers in the unopened bolls.

From the field material 1200 flowers were marked with dated tags in one day during the period of profuse blooming. Thereafter, until the time of dehiscence, 30 of the bolls were collected daily and from these ten representative ones were chosen for measurement. The bolls were collected at an early hour every morning in order to avoid variations due to loss of water during the warmer periods of the day. The measurements followed quickly in the laboratory. The weight and volume (by displacement of water) were first obtained for the ten entire bolls. Dissection was carried out under conditions which prevented drying and the weights and volumes of the walls and partitions measured separately.

Plants of the Pima and Acala varieties as well as Super Seven were grown in the greenhouses of the Boyce Thompson Institute during the summer of 1932. Cultural experiments with seeds planted at monthly intervals during the previous year had furnished information concerning the necessary requirements for the growth of the plants under greenhouse conditions in this particular climate. Plants grown from seeds which were germinated in March and were ready for transplanting early in April were well adapted to the temperatures and day-lengths of the ensuing months. The seeds were delinted in concentrated sulphuric acid, washed, and treated for 10 minutes with a solution of HgCl_2 (1/1000). Germination in individual pots containing sterilized soil was followed by the transplanting of the 3 to 4-inch seedlings to 14-inch, unglazed, earthenware pots. At the end of the first month after transplanting 10 grams of 4-8-7 commercial fertilizer were added to each pot weekly. The greenhouse temperature was maintained at about 75° F. and the plants were arranged in a way which afforded well distributed illumination. Weights and volumes of the entire

² Field and laboratory facilities were furnished through the courtesy of H. W. Barre and G. M. Armstrong. Valuable assistance was given by Bertram Barre in the collection and measurement of the material.

TABLE I
COUNTS OF FIBER ABNORMALITIES IN MICROSCOPIC MOUNTS OF THREE VARIETIES
OF COTTON FIBERS

Pima	Super Seven	Acala
10	117	67
23	119	162
20	68	118
11	118	179
16	80	162
6	71	242
20	99	167
17	84	249
20	103	195
9	77	173
Average 15.2	93.6	171.4

and of the dissected bolls were taken according to the methods used previously for field material. The number of bolls available from the greenhouse was necessarily much less than from the field. A greater uniformity in size and weight of bolls of the same age offset, however, this quantitative disadvantage. A comparison of boll development in Super Seven cotton in the field and in the greenhouse indicates the value of greenhouse-grown

TABLE II
AVERAGE WEIGHTS AND VOLUMES OF ENTIRE AND DISSECTED BOLLS OF
GREENHOUSE-GROWN PIMA COTTON

Days after flowering	Weight of bolls in grams	Volume of bolls in cc.	Weights of walls and partitions in grams	Volume of walls and partitions in cc.	Weight of seeds and fibers in grams	Volume of seeds and fibers in cc.
4	0.40	—	0.30	0.35	0.09	0.06
7	1.00	1.00	0.65	0.70	0.30	0.40
11	2.82	3.00	1.70	1.90	1.02	1.50
12	3.10	3.30	1.80	1.98	1.20	1.60
14	4.70	6.00	2.60	2.50	2.02	2.50
15	5.00	5.00	2.82	2.60	2.10	2.00
19	9.34	10.00	4.92	4.80	4.30	5.00
20	11.47	12.50	6.22	6.10	5.10	6.00
22	12.00	13.00	6.80	7.50	5.10	6.30
23	15.35	16.50	7.45	8.00	7.70	8.80
24	14.50	16.00	7.10	7.50	7.45	8.40
25	13.15	13.80	6.55	7.00	6.45	8.00
26	15.60	16.00	8.10	9.00	7.40	8.50
27	15.50	15.80	8.44	8.60	6.67	7.90
29	16.12	18.00	8.17	9.00	7.90	9.00
33	16.40	18.00	7.92	9.00	8.37	9.00
36	16.15	17.00	8.20	8.50	7.90	9.00
37	17.00	18.00	7.92	9.00	8.95	10.00
41	16.25	18.00	7.75	8.00	8.20	9.50
43	16.62	18.00	7.92	8.50	8.60	9.50
46	16.50	18.00	7.50	8.20	8.95	10.00
48	15.80	16.00	6.90	7.50	8.95	9.50
50	14.45	16.00	6.85	7.20	7.50	9.50
59	13.00	14.00	6.00	7.00	6.90	8.00

TABLE III
AVERAGE WEIGHTS AND VOLUMES OF ENTIRE AND DISSECTED BOLLS OF FIELD-GROWN AND GREENHOUSE-GROWN SUPER
SEVEN COTTON

Days after flower- ing	Weight of bolls in grams		Volume of bolls in cc.		Weight of walls and partitions in grams		Volume of walls and partitions in cc.		Weight of seeds and fibers in grams		Volume of seeds and fibers in cc.	
	Field	Green- house	Field	Green- house	Field	Green- house	Field	Green- house	Field	Green- house	Field	Green- house
1	0.16		0.20		0.13		0.15		0.03		0.03	
2	0.38		0.43		0.34		0.36		—		0.07	
3	0.54		0.68		0.44		0.55		0.05		0.10	
4	0.73		0.95		0.59		0.70		0.12		0.20	
5	1.22		1.35		0.84		0.99		0.34		0.49	
6	1.64		1.87		1.17		1.17		0.50		0.67	
7	2.51		3.15		1.61		1.72		0.85		1.02	
8	3.61		3.90		2.26		2.27		1.30		1.61	
10	0.22		0.90		3.80		3.85		2.32		2.70	
11	9.00		9.80		5.28		4.80		3.52		4.00	
12	10.04		12.50		6.21		6.20		4.53		5.20	
13	11.82	12.05	12.80	13.50	6.70	5.70	6.80	6.00	4.83	6.12	5.00	7.00
15	16.26	12.70	17.80	14.00	8.31	5.85	8.40	6.50	7.55	6.82	8.60	7.50
17	20.31		22.50		10.44		10.80		9.42		11.02	
19	23.19		24.50		11.42		11.50		11.50		13.00	
21	24.42		26.20	18.00	12.23	8.48	12.50	9.00	11.77	7.95	13.00	8.50
24	25.85		27.50		12.53		12.50		13.11		15.00	
26	23.20		24.00		10.54		11.50		11.94		12.75	
28	24.58		26.00		11.71		12.00		12.76		14.50	
29		23.55	25.00	25.00		10.02		11.00		13.35	15.00	
32	22.65		24.00		10.50		11.00		11.99		14.20	
35	25.57		27.00		12.25		13.00		13.09		15.00	
38	23.79		24.50		10.82		11.00		12.75		14.00	
40	22.83		24.00		10.56		11.00		11.89		14.00	
41		24.35	26.00	26.00		10.75		11.00		13.39	15.50	
42	21.60		23.50		9.60		10.00		11.81			
45	20.81	25.72	23.00	28.00	8.88	10.92	10.00	11.20	11.64	14.57	12.50	16.00
48	12.36	27.02	14.50	30.00	5.37	12.00	6.00	12.50	7.01	15.00	16.00	18.00
52		28.12	30.00	30.00		11.90		12.00		16.12	9.50	
55		18.45	19.00	19.00		8.25		8.50		10.00		19.00

cotton plants for experimental material (Table III). In all phases of boll development, the greenhouse material was found to be fully the equal of that grown in the field and, in many respects, it was superior.

The counts of abnormalities in microscopic mounts of the three varieties were made from greenhouse material. A considerable amount of practice was necessary in order to standardize the quantity of fibers taken in each instance. The tufts were separated from the seed coat with a small, sharp blade ground especially for the purpose. This method of removal

TABLE IV
AVERAGE WEIGHTS AND VOLUMES OF ENTIRE AND DISSECTED BOLLS OF
GREENHOUSE-GROWN ACALA COTTON

Days after flowering	Weight of bolls in grams	Volume of bolls in cc.	Weight of walls and partitions in grams	Volume of walls and partitions in cc.	Weight of seeds and fibers in grams	Volume of seeds and fibers in cc.
5	0.93	1.00	0.70	0.80	0.22	0.40
8	1.19	2.20	0.85	1.50	0.32	0.50
10	2.30	2.43	1.55	—	0.75	0.80
13	5.00	5.20	2.32	3.00	2.05	3.50
16	10.40	11.20	4.92	5.20	5.32	7.00
19	20.70	20.10	8.40	8.00	11.90	13.00
21	27.55	30.00	11.07	12.00	16.05	18.00
22	26.10	30.00	11.15	12.00	14.67	19.00
23	29.62	33.00	11.40	12.00	18.00	22.00
25	31.24	35.00	12.60	12.20	18.54	22.00
27	31.95	35.00	12.95	12.00	18.75	23.00
30	32.80	39.00	12.82	13.00	19.70	25.00
34	32.15	38.00	12.85	13.00	19.10	25.00
36	35.22	37.00	13.70	15.00	21.30	26.00
38	37.72	42.50	15.12	16.00	22.30	28.00
43	39.42	43.00	15.10	17.00	24.22	30.00
47	37.50	43.00	15.10	16.00	22.10	28.00
50	37.52	42.50	14.74	14.00	22.72	28.00
54	24.00	—	8.25	9.60	15.60	25.00

eliminated the possibility of producing artificial abnormalities along the fiber axis through crushing. In expressing the results, the single seed and not the count from each microscopic mount was taken as a unit. The value used in each instance represents, therefore, the counts from three separate mounts taken from the proximal, median, and distal portions of a seed.

In order to avoid error in interpretations, minor abnormalities, such as change in direction of growth, unaccompanied by enlargement, or slight changes in diameter along the fiber axis, were not counted. Change in direction of growth accompanied by enlargement, branching, gigantomorphism, or localized attenuation are, on the other hand, unmistakable.

RESULTS

The 30 seeds which were used for abnormality counts shown in Table I were taken in every instance from the second position in the locule at the

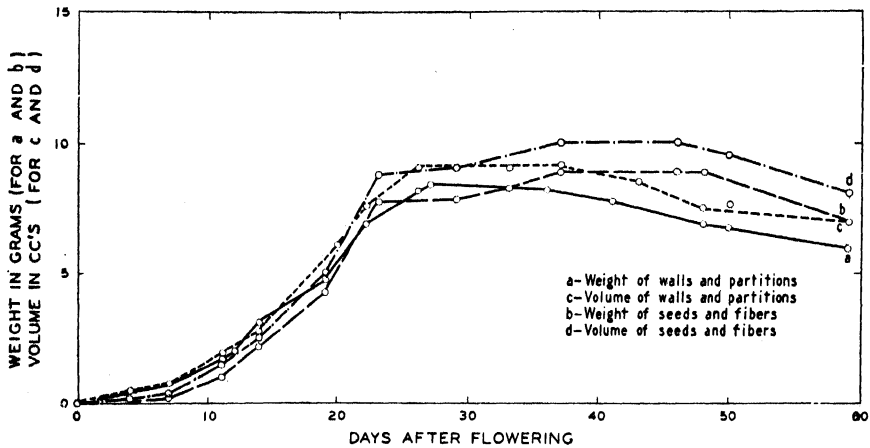


FIGURE 1. Weight-volume relationships in parts of developing Pima bolls.

distal end of the boll. Large numbers of counts made by several different methods indicated about the same range of variation in fibers of the same variety. Different sets of counts fluctuated to some extent in the neighborhood of the average values shown. Since the general relationships with respect to the numbers estimated by any method were the same, however, these approximations are believed to represent adequately the presence of large numbers of abnormalities in Acala, an intermediate number in Super Seven, and a small number in Pima (Table I).

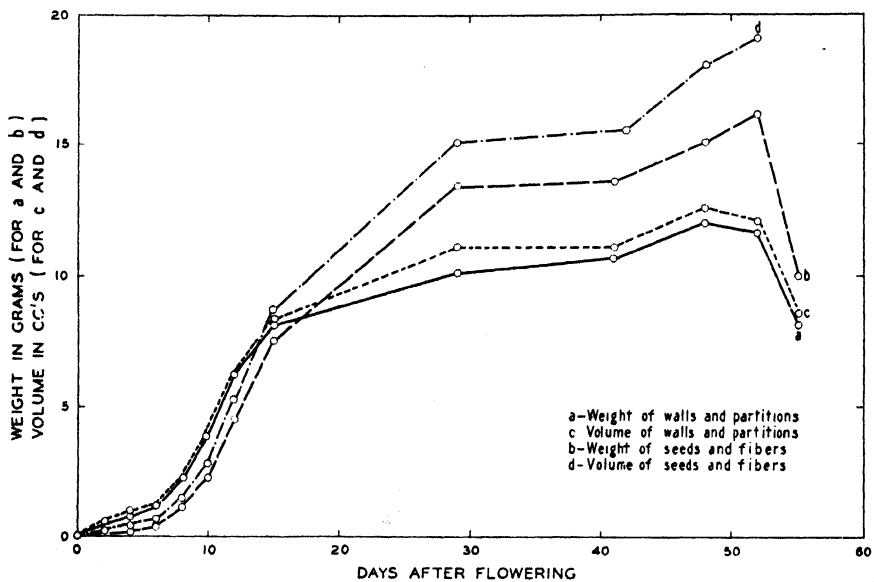


FIGURE 2. Weight-volume relationships in parts of developing Super Seven bolls.

The weights and volumes of the seeds and fibers and of the walls and partitions in Pima, Super Seven, and Acala are given in Tables II, III, and IV respectively. A graphic representation of the same data is given in Figures 1, 2, and 3. Many of the details of boll development discussed earlier upon the basis of general observations are expressed again in a more specific form. In every one of the three varieties the development of walls

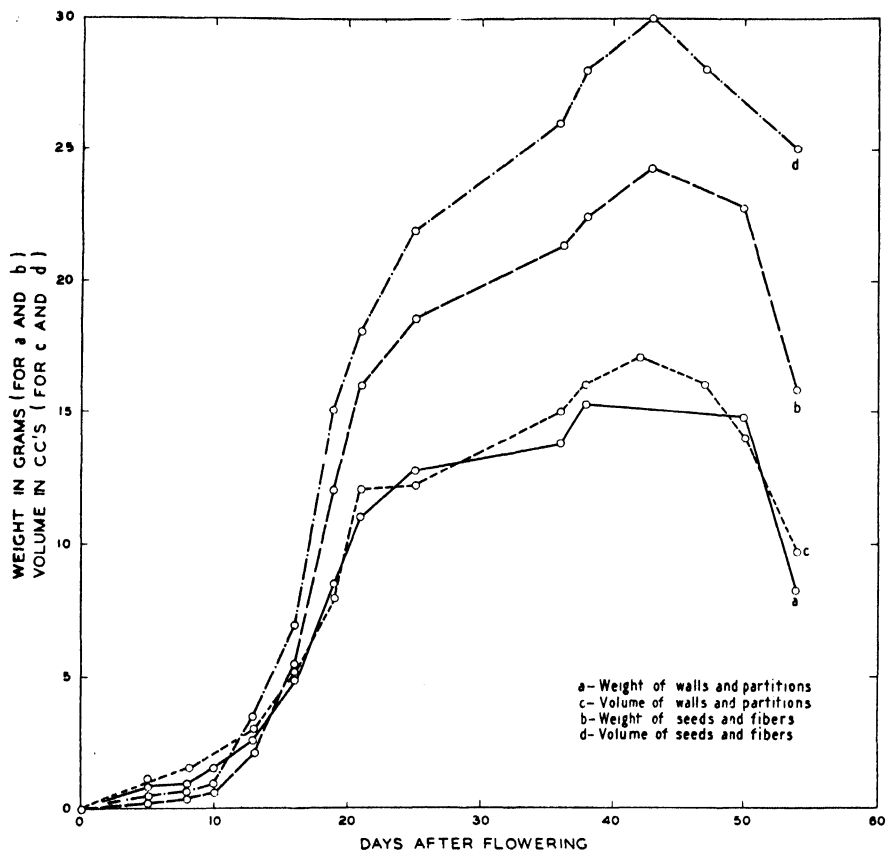


FIGURE 3. Weight-volume relationships in parts of developing Acala bolls.

and partitions proceeded rapidly until about the twentieth day and much more slowly for the next twenty or twenty-five days. At the time of dehiscence there was a rapid decline in both weight and volume of walls and partitions due to drying.

The relative weights and volumes of seeds and fibers on the other hand present sharp contrasts previous to as well as after the time of cessation of wall enlargement. In Pima the increase in weights and volumes of seeds and fibers proceeded very slowly. The curves for walls and partitions and seeds and fibers diverge only slightly at any period as shown in Figure 1.

The same curves for Super Seven have a much wider divergence as shown in Figure 2. It is in Acala, however, that the curves deviate to the greatest extent (Fig. 3). The comparative divergence of these curves based upon values for wall and partition and seed and fiber development is believed to represent only another form of expression of the relatively loose arrangement of fibers in Pima, the more compact arrangement in Super Seven, and the very dense arrangement in Acala. Since the gradient for increase in density of the fiber mass extends in the same direction as that for increase in number of abnormalities, $\text{Pima} < \text{Super Seven} < \text{Acala}$, a definite relationship is suggested between these two factors.

The application of this principle may be discussed in terms of the single fiber. Cell enlargement and the formation of a thick cell membrane are two of the more conspicuous phases of cotton fiber growth. The tendency to enlarge in an approximately linear direction is obviously inherent. If obstructed in one region of the boll cavity, however, the portion of the fiber concerned apparently appropriates any available space in the immediate vicinity for enlargement. This may result in change in diameter, change in direction of growth, or any one of the many different types of abnormalities. Repeated adaptation to such conditions may produce even in a single fiber a large number of abnormalities of different types placed at indefinite intervals along the fiber axis. The need for such adaptation upon the part of a single fiber and consequently the probability of abnormality formation would be greater with increasing density of the fiber mass.

SUMMARY

Few abnormalities are found in the fibers of Pima, Super Seven, and Acala cotton during the period of development in which increase in size of the boll cavity is keeping pace with the enlargement of the fiber mass.

Counts of abnormalities from mature bolls of the three varieties show a large number in Acala, a smaller number in Super Seven, and very few in Pima.

Upon the basis of the relative weights of walls and partitions and seeds and fibers throughout the entire period of development, a lesser density of the fiber mass is shown in Pima, a greater density in Super Seven, and a much greater density in Acala.

Since the gradient for increase in density of the fiber mass extends in the same direction as that for increase in number of abnormalities, it is suggested that the density of the fiber mass within the boll during the later stages of development is one of the important factors in the determination of the number of fiber abnormalities found in these three varieties of cotton.

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FUNGICIDAL ACTION AND THE PERIODIC SYSTEM OF THE ELEMENTS¹

S. E. A. MCCALLAN AND FRANK WILCOXON

The elements which have found most frequent application as fungicides, either in the elementary state or as compounds, are copper, sulphur, and mercury. In spite of a large number of experiments dealing with compounds of other elements, a systematic study of fungicidal action as related to the position of the elements in the periodic system does not appear to have been made.

Wöber, in 1920 (82), reviewed the work of a number of investigators, and attempted to trace certain relationships among the metals forming fungicidal compounds, when specific gravity was plotted against atomic weight. However, in this study, the results of many investigators were used, and the experimental conditions were different in the various experiments considered. Kotte (41), using spores of *Plasmopara viticola*, and Burström (11), using those of *Tilletia tritici*, tested the relative toxicity of a number of salts. Clark (14) and others (6, 20, 23, 43, 44, 83) have also made comparisons of the effect of certain salts on the germination of spores, or growth of various fungi. Few of these investigators attempted any correlation of toxicity with position in the periodic system, and some have failed to consider the possibility of adsorption or precipitation of the salts by the media on which the fungi were grown. Kunkel (43, 44) has pointed out the variation in toxicity that may result by the addition of different nutrient substances.

In the present work an attempt has been made to compare compounds of a large number of elements with regard to their effect on the germination of fungous spores. It was hoped that such a study might show definite periodic relationships among the elements as regards toxicity, and might also indicate the fungicidal value of elements now little used. The compounds were tested under as nearly the same conditions as possible, and spores of several species of fungi were used, in order to see to what extent the conclusions drawn might be of general application.

There are a number of difficulties involved in such a study. The chemical properties of the elements vary according to their position in the periodic system, so that a compound of an element which is quite suitable for the purpose of testing in one part of the system may have no counterpart in another portion of the system. Compounds of many elements undergo hydrolysis in the presence of water, forming acid or alkaline solutions, and sometimes insoluble products, so that it is not possible to

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use corresponding compounds throughout. Furthermore, many elements, notably the non-metals, may be quite toxic in certain combinations and non-toxic in others. Nevertheless, in spite of these difficulties, it is possible to show that there are several centers of toxicity in the periodic table, and that toxicity declines more or less regularly as we move away from these toxic centers.

METHODS

The method of testing has been described in previous publications (46, 47, 48, 49, 78, 79), and consists essentially in suspending the spores in drops of the solution to be tested and recording the percentage germination after 20 to 24 hours. Four species of fungi were used, namely *Sclerotinia americana* (Worm.) Nort. & Ezek., *Botrytis paeoniae* Oud., *Pestalotia stellata* B. & C., and *Uromyces caryophyllinus* (Schr.) Wint. Conidia of the first three species were obtained from 5- to 10-day-old cultures growing on potato dextrose agar at 20° C., while the uredospores of *Uromyces* were produced on naturally infected greenhouse carnations. Tests with the former species were performed at room temperatures with an average variation per test of from 23° to 25° C. The variation in the *Uromyces* tests was 19° to 24° C.

The authors (80) have found that the addition of a small amount of orange juice will markedly stimulate the germination of certain fungous spores, and since the spores of *Botrytis* and *Pestalotia* often germinate irregularly, orange juice was added in these cases. In the case of *Botrytis* the final concentration of filtered juice was 0.02 per cent, and in the case of *Pestalotia* 0.05 per cent. Under these conditions a germination of 99 to 100 per cent was obtained in the controls. In each experiment about 300 spores were counted at each concentration tested, and each experiment was repeated from one to four times.

The compounds tested were of C. P. grade and were generally used without further purification.

The experimental data were plotted in the form of a toxicity curve showing the relation between percentage germination and concentration. Such curves are usually sigmoid in form, and from inspection of the curve, the concentration which permits 50 per cent germination or the LD 50 may be estimated. The LD 50 in this case does not necessarily imply the concentration killing 50 per cent of the spores but the concentration permitting a 50 per cent response. The particular response measured in this case is the ability to germinate in the presence of the toxic agent. This value may be taken as a measure of the toxicity of the particular compound under consideration. However, if it is desired to learn what degree of significance to attach to this estimation, the method described by Gaddum (28) may be used. Here the concentrations are plotted logarithmically, while the percentage germination values are transformed to units

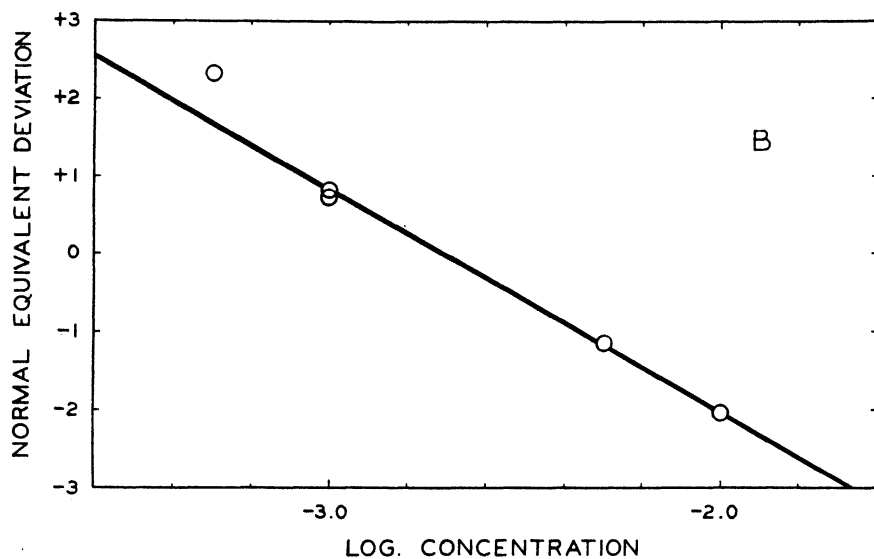
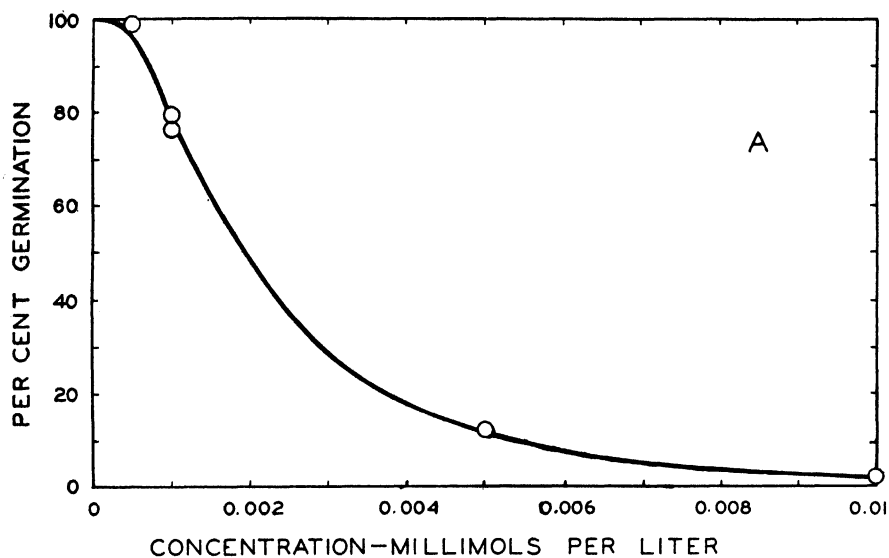


FIGURE 1. Toxicity curves for osmium tetroxide on *Botrytis paeoniae*. A. Sigmoid curve obtained by usual method of plotting. B. Same data plotting normal equivalent deviations against logarithms of concentration and with fitted regression line.

called by Gaddum "normal equivalent deviations." Plotted in this way, the results lie more or less on a straight line, and a straight line may be fitted to the data by the method of least squares, giving the proper weight to the different determinations. The concentration at which this line crosses the axis of abscissas gives the logarithm of the dose corresponding to 50 per cent germination.

For example, Figure 1 shows the toxicity data for osmium tetroxide, plotted in this way with the regression line fitted to the data. The logarithm of the concentration permitting 50 per cent germination is -2.72 corresponding to a concentration of 0.0019 millimol per liter. The equation of the line is:—

$$y = \bar{y} + b(x - \bar{x})$$

\bar{y} is the mean value of y , and b is the slope. Distances along the axis of x are measured from mean x . Deviations in \bar{y} may be obtained by measuring the vertical distance of each point from the line, and the standard deviation may be calculated in the usual way from the sums of the squares of these deviations. Thus:—

$$\sigma_{\bar{y}}^2 = \frac{\sum ([y - \{\bar{y} + b(x - \bar{x})\}]^2 \times Bn)}{(n-1) \times \sum Bn}$$

Bn is the weight to be attached to each observation (28). Deviations in the slope, b , are equal to the deviations of \bar{y} each divided by its corresponding $(x - \bar{x})$.

Hence the formula for σ_b^2 is as follows:

$$\sigma_b^2 = \sum \frac{\left(\left[\frac{y - \{\bar{y} + b(x - \bar{x})\}}{x - \bar{x}} \right]^2 \times Bn(x - \bar{x})^2 \right)}{(n-1) \times \sum [Bn(x - \bar{x})^2]}$$

It may be seen then that σ_b^2 is related to $\sigma_{\bar{y}}^2$ as follows:—

$$\sigma_b^2 = \sigma_{\bar{y}}^2 \times \frac{\sum Bn}{\sum [Bn(x - \bar{x})^2]}$$

The value of $(x - \bar{x})$ at which y is 0, that is at 50 per cent germination, is $-\bar{y}/b$. Therefore its standard deviation may be obtained from the formula for the standard deviation of a quotient (53, p. 259).

RESULTS

The values of the 50 per cent lethal dose or LD 50 are shown in Table I, expressed in millimols per liter. Only the toxic elements (average LD 50 of less than 1.0) are included.

In order to give a graphic picture of the relative toxicity of the various elements as related to their position in the periodic system, the reciprocal

of the LD 50 was used to obtain the heights of the columns shown in Figures 2 to 5. The form of the periodic system presented is that of Werner and Pfeiffer as given by Remy (64, p. 11). Here the height of the column

TABLE I

CONCENTRATIONS PERMITTING 50 PER CENT GERMINATION (LD 50), IN MILLIMOLS PER LITER, OF THE TOXIC ELEMENTS* LISTED IN ORDER OF AVERAGE TOXICITY

Compounds of	<i>Sclerotinia americana</i>	<i>Botrytis paeoniae</i>	<i>Pestalotia stellata</i>	<i>Uromyces caryophyllinus</i>
Silver	0.00043	0.0012	0.00060	0.00040
Osmium	0.00027	0.0019	0.0083	0.00067
Mercury	0.0034	0.0036	0.0026	0.0056
Cerium	0.0045	0.0030	0.022	0.013
Ruthenium	0.0038	0.0048	0.019	0.021
Lanthanum	0.0043	0.0030	0.028	0.022
Cadmium	0.0046	0.0058	0.033	0.027
Uranium	0.0069	0.015	0.48	0.0036
Copper	0.0062	0.022	0.027	0.021
Yttrium	0.016	—	—	—
Erbium	0.023	0.012	0.038	0.011
Neodymium	0.043	0.0091	0.037	0.015
Lead	0.010	0.0074	0.031	0.089
Gold	0.085	0.024	0.030	—
Thallium	0.026	0.015	0.25	0.038
Aluminum	0.81	0.030	0.25	0.024
Thorium	0.31	0.032	0.11	0.15
Palladium	0.15	0.39	0.30	0.025
Manganese	0.16	0.32	0.065	—
Zinc	0.13	0.21	0.24	0.14
Nickel	0.058	0.45	0.40	0.14
Cobalt	0.16	0.40	0.14	0.68
Chromium	0.0066	0.0091	0.16	> 1.00
Arsenic	0.013	0.24	0.037	> 1.00
Platinum	0.14	> 0.10	0.19	—
Tantalum	0.26	0.32	0.32	—
Vanadium	> 1.00	0.13	0.20	—
Titanium	0.60	> 1.00	0.32	—

* Exclusive of the sulphur group and the halogens. The toxicity of the volatile hydrides was not considered in obtaining the above values.

is a measure of the toxicity of the element. The average probable error of the mean for these values is 12 per cent of the mean. For elements having an LD 50 greater than 0.1 millimol no height is indicated. In these figures the periodic table is shown rotated 180° from the conventional position in order that the heights of the columns may be seen more clearly.

THE ALKALIES AND ALKALINE EARTHS

The following elements were tested in the form of chlorides: Li, Na, K, Rb, Ca, Sr, Ba. Three species of fungous spores were used, namely *Sclerotinia americana*, *Botrytis paeoniae*, and *Pestalotia stellata*. The toxicity was negligible in every case at concentrations of 1.0 millimol per liter or less.

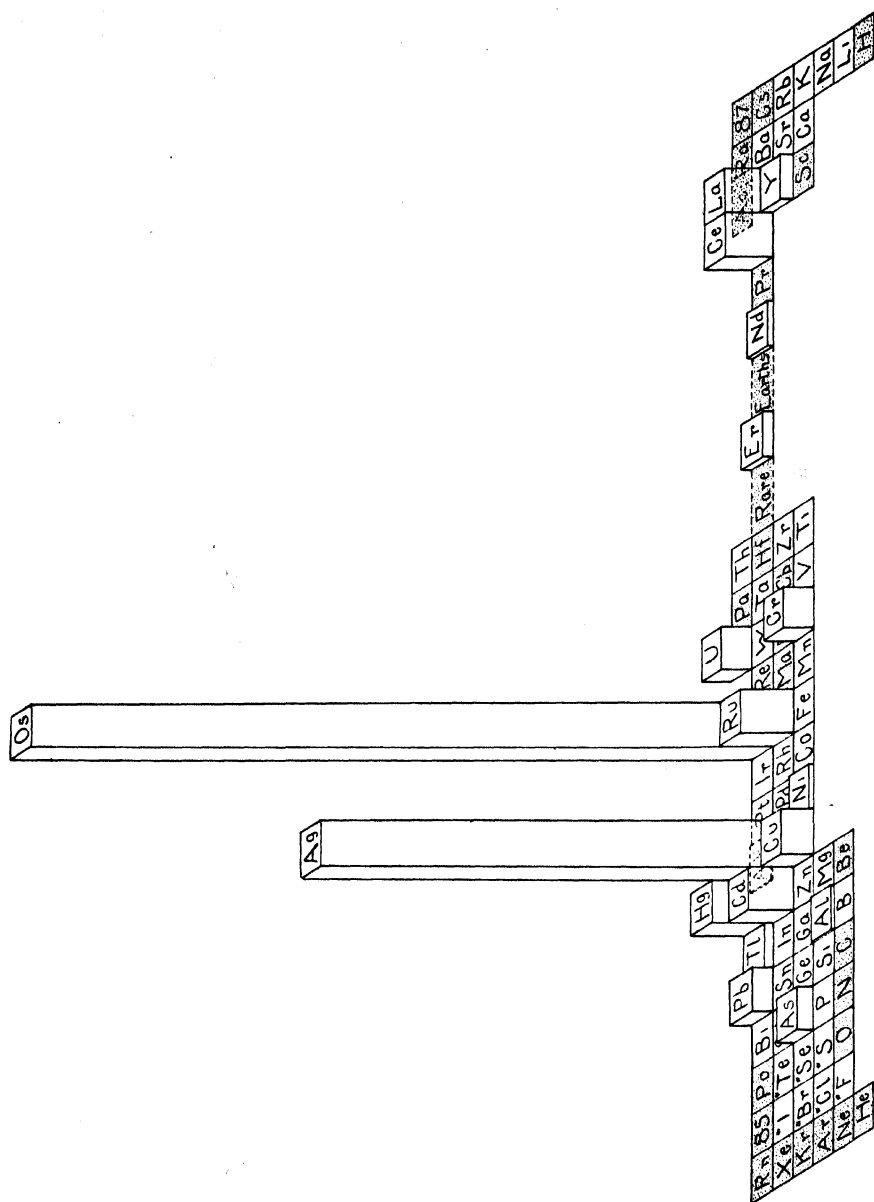


FIGURE 2. Toxicity of compounds of the elements toward conidia of *Sclerotinia americana*, in relation to the position of the elements in the periodic system. The heights of the columns are the reciprocals of the LD 50. Stippled elements were not tested and starred elements can be assigned no definite value since the toxicity varies with the degree of oxidation.

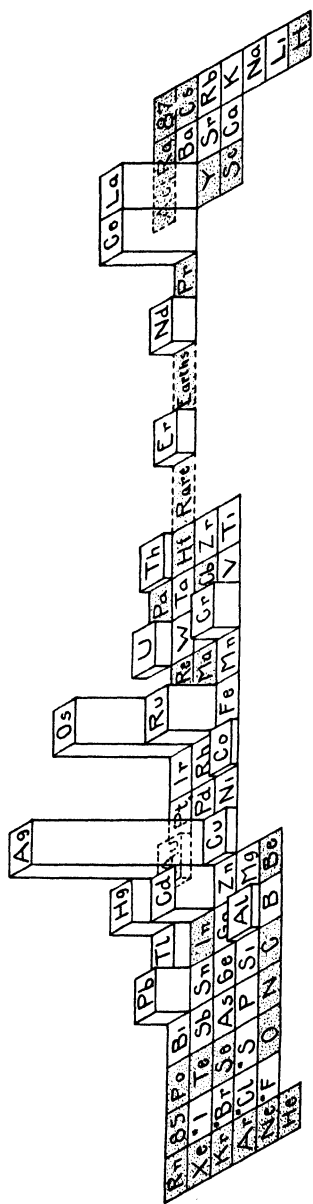


FIGURE 3. Toxicity of compounds of the elements toward conidia of *Batrytis paeoniae*, in relation to the position of the elements in the periodic system. The heights of the columns are the reciprocals of the LD 50. Stippled elements were not tested and starred elements can be assigned no definite value since the toxicity varies with the degree of oxidation.

YTTRIUM, LANTHANUM, AND THE RARE EARTHS

Yttrium and lanthanum, as well as cerium, neodymium, and erbium were found to exhibit marked toxicity toward the fungi tested. Yttrium was tested as nitrate, $\text{Yt}(\text{NO}_3)_3 \cdot 4\text{H}_2\text{O}$, using conidia of *Sclerotinia americana*; lanthanum as chloride, $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$, was toxic to all four fungi, as was cerium sulphate, $\text{Ce}_2(\text{SO}_4)_3 \cdot 8\text{H}_2\text{O}$, neodymium chloride, $\text{NdCl}_3 \cdot 6\text{H}_2\text{O}$, and erbium chloride, $\text{ErCl}_3 \cdot 6\text{H}_2\text{O}$. The toxicity of cerium exceeded that of copper to the four fungi used. Monazite sand, which is a commercial source of the rare earths, was found to be non-toxic when slides were dusted with the powdered material, and drops of spore suspension subsequently placed on them to germinate.

Discussion. Both Burström (11) and Dessy (20) reported cerium among the more toxic elements. During the world war and more recently rare earth salts have been used in Central Europe as a substitute for Bordeaux mixture under the name "Perocide" (3, 32). This preparation is said to be a mixture of rare earth sulphates and to contain 45 per cent of cerium oxide. The "vivatex" process of rendering cotton fabrics mildew-proof is based on the use of cerium and certain other rare earths (84).

Since the elements of this group are so generally toxic, the more common ones, cerium, yttrium, and lanthanum, offer promise of a more general use as fungicides.

TITANIUM, ZIRCONIUM, HAFNIUM, AND THORIUM

Of these four elements titanium was tested as potassium fluotitanate, $\text{K}_2\text{TiF}_6 \cdot \text{H}_2\text{O}$, zirconium as zirconium oxychloride, $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$, and thorium as nitrate, $\text{Th}(\text{NO}_3)_4 \cdot 12\text{H}_2\text{O}$. Thorium nitrate showed slight toxicity to all four fungi, but the other elements were non-toxic.

VANADIUM, COLUMBIUM, AND TANTALUM

Vanadium was tested as potassium vanadate, K_3VO_3 , and tantalum as potassium fluotantalate, K_2TaF_6 . Neither of these salts was toxic.

CHROMIUM, MOLYBDENUM, TUNGSTEN, AND URANIUM

Chromium was tested as potassium chromate, K_2CrO_4 , potassium dichromate, $\text{K}_2\text{Cr}_2\text{O}_7$, and as chrome alum, $\text{K}_2\text{SO}_4 \cdot \text{Cr}_2(\text{SO}_4)_3 \cdot 24\text{H}_2\text{O}$; molybdenum as sodium molybdate, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; and tungsten as sodium tungstate, $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$. Uranium was tested as uranyl acetate, $\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$. Chromium showed marked toxicity to *Sclerotinia* and *Botrytis*, its action being comparable to that of copper, but in the case of the other two fungi the toxicity was negligible. In the case of *Sclerotinia*, *Botrytis*, and *Pestalotia*, the toxicity of chromium was practically the same, regardless of the form in which it was used. Molybdenum and tungsten were non-toxic to *Sclerotinia*, *Botrytis*, and *Pestalotia*. In the case of *Pesta-*

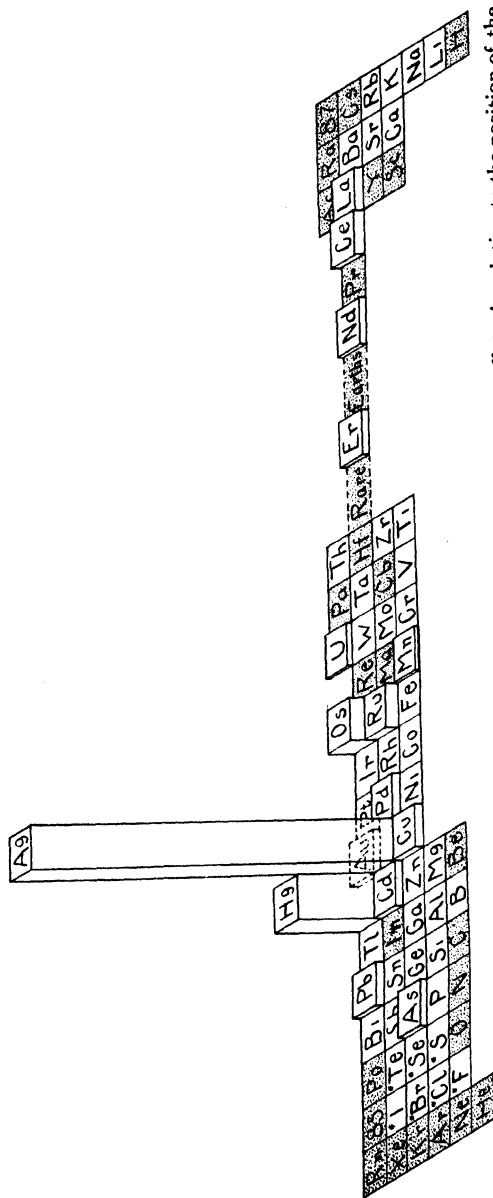


FIGURE 4. Toxicity of compounds of the elements toward conidia of *Pestalotia stellata*, in relation to the position of the elements in the periodic system. The heights of the columns are the reciprocals of the LD 50. Stippled elements were not tested and starred elements can be assigned no definite value since the toxicity varies with the degree of oxidation.

lotia stellata an unusual swelling of the germ tube to form a large spherical vesicle was invariably observed in 1.0 millimolar solutions of sodium tungstate. This phenomenon is illustrated in Figure 6. The photograph was taken after 24 hours in the solution beyond which time there is no appreciable change. Uranium was as toxic as copper to three of the fungi, while in the case of *Uromyces* it was much more toxic than copper, and was only exceeded by silver and osmium.

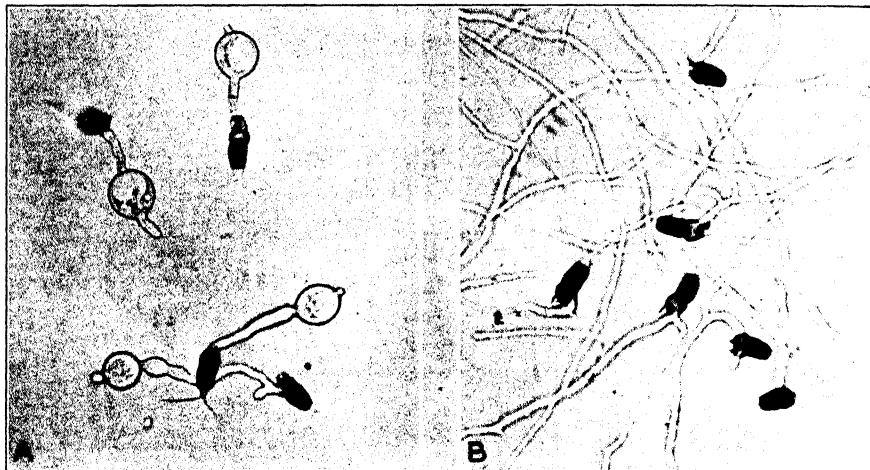


FIGURE 6. A. Specific response of germ tubes of *Pestalotia stellata* in 1.0 millimolar solution of sodium tungstate. $\times 333$. B. Control. $\times 333$.

Discussion. Clark (14) found that chromium compounds were exceeded in toxicity only by mercury and silver, while Kotte (41) and Bateman and Baechler (6) found that they were intermediate in this respect. The latter consider that sodium chromate offers promise as a wood preservative, while in tests, Kress, *et al.* (42) found that sodium dichromate prevented the decay of wood pulp. Calcium and potassium dichromates gave good control of wheat bunt in the Urals (19). Since the present authors' experiments indicate a wide variation in sensitivity to chromium among the fungi tested it is possible that these compounds may find further application as fungicides against specific fungi.

MANGANESE, MASURIUM, AND RHENIUM

Only the first named was tested, as potassium permanganate, KMnO_4 . It was not toxic to *Sclerotinia* and *Botrytis*, and showed only slight toxicity to *Pestalotia*.

Discussion. In higher concentrations than tested above, potassium permanganate has been employed as a disinfectant for soils, rhizomes, cut-

tings, fruits, etc., and as a spray to eradicate powdery mildews, particularly that of the grape in France (72).

IRON, RUTHENIUM, AND OSMIUM

Iron tested as $\text{Fe}(\text{SO}_4)_3 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$, ferric alum, was not toxic. Ruthenium was tested in the form of "ruthenium red," $\text{Ru}(\text{OH})_2\text{Cl}_4 \cdot 7\text{NH}_3 \cdot 3\text{H}_2\text{O}$, while osmium was tested as osmium tetroxide, OsO_4 . Both these compounds were highly toxic, OsO_4 being the more toxic of the two.

Discussion. Although osmium and ruthenium exhibit high toxicity in these tests, it is questionable if they could find practical use as fungicides due to their cost.

COBALT, RHODIUM, AND IRIDIUM

Cobalt as acetate, $\text{Co}(\text{C}_2\text{H}_3\text{O}_2) \cdot 4\text{H}_2\text{O}$, rhodium as chloride, RhCl_3 , and iridium as IrCl_4 were not appreciably toxic.

NICKEL, PALLADIUM, AND PLATINUM

Nickel chloride, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, showed only feeble toxicity, far inferior to that of copper. Palladous chloride, PdCl_2 , and chloroplatinic acid, $\text{H}_2\text{PtCl}_6 \cdot \text{H}_2\text{O}$, showed only slight toxicity.

Discussion. Several investigators reporting on the comparative toxicity of various metal salts to particular fungi find that nickel is at least equal in toxicity to copper (41) if not greater (6, 11, 14, 20, 23), while Kotte (41) alone finds it equal to mercury. A few years ago a number of tests were reported on the use of nickel compounds as seed disinfectants, especially for cereal smuts. The results were conflicting and in general were not promising (27, 37, 45, 51, 52, 70).

COPPER, SILVER, AND GOLD

Copper was tested as chloride, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and acetate, $\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$. These salts were, as might be expected, very toxic, but it is worth noting that copper is exceeded in toxicity by six or seven other elements in the periodic system. No significant difference in toxicity was found between copper chloride and copper acetate. Silver was tested as nitrate, AgNO_3 , sulphate, Ag_2SO_4 , and acetate, $\text{AgC}_2\text{H}_3\text{O}_2$. It was found to be the most toxic element of all to three of the fungi, and in the case of *Sclerotinia* was exceeded only by osmium. As in the case of copper, no significant difference in toxicity was found between the compounds of silver tested. Gold which was tested as chlorauric acid, $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$, was much less toxic than silver, but was about equal to copper in the case of *Botrytis* and *Pestalotia*.

Discussion. Although Kotte (41) reported copper the most toxic element together with mercury and nickel, and silver only of moderate toxicity, other investigators (6, 11, 14, 20, 44, 83) find copper is exceeded in

toxicity by various elements including silver. Silver, however, was less toxic than mercury. Clark (15) and also Kotte (41) have observed much the same toxicological values for the different salts of copper.

Silver nitrate has been used successfully as a seed disinfectant for *Helianthus*, *Cannabis*, *Cucumis*, *Secale*, and *Zea* (63), and in the control of brown-patch was equal to mercury salts (54). However, because of expensiveness silver compounds will be limited probably to small scale treatments such as the recommended disinfection of tobacco seed with silver nitrate to control *Bacterium tabacum* (38) and *Phytophthora polycolora* (13), though a recent paper (10) describes the use of silver in extremely low concentrations for the sterilization of water.

BERYLLIUM, MAGNESIUM, ZINC, CADMIUM, AND MERCURY

Beryllium was not tested. Magnesium as chloride, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, showed no appreciable toxicity. The remaining three elements showed increasing toxicity with increase in atomic weight. Zinc as chloride, ZnCl_2 , and as acetate, $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$, showed slight toxicity. Cadmium as chloride, $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$, and acetate, $\text{Cd}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$, was more toxic than copper to *Botrytis* and *Sclerotinia* and about equal to copper in the case of *Pestalotia* and *Uromyces*. Mercury as chloride, HgCl_2 , and acetate, $\text{Hg}(\text{C}_2\text{H}_3\text{O}_2)_2$, was highly toxic to all the fungi tested.

Discussion. Investigators who have made comparisons of zinc, cadmium, and mercury find zinc exhibiting but little toxicity, and cadmium intermediate (6, 14) or else zinc approximately equal to cadmium but both less than mercury (11, 20, 41, 44) while all agree mercury is one of the most toxic, if not the most toxic element.

Zinc chloride has been employed extensively as a wood preservative (22) and has given good results in the treatment of fire blight cankers (18, 50). Recent experiments indicate the suitability of a zinc-lime spray for the control of bacterial and possibly also fungous diseases of the peach (65) and to zinc oxide as an effective seed and soil disinfectant for damping-off (35).

Cadmium compounds have apparently received but little attention as fungicides, other than the patenting of cadmium sulphate and chloride as dip treatments to prevent the moulding of fresh fruits by *Penicillium* spp. (4), though Bateman (5) considers cadmium sulphate a very satisfactory wood preservative in all respects except its prohibitive cost. Further studies should be made of cadmium to determine its practical application, among its possibilities being that of a substitute for mercury.

BORON, ALUMINUM, GALLIUM, INDIUM, AND THALLIUM

Boron was tested as boric acid, H_3BO_3 ; aluminum as potassium alum, $\text{Al}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$; gallium and indium as chlorides prepared from

the metals; and thallium as thalious sulphate, Tl_2SO_4 . Thallium was the most toxic element of this group, being comparable to copper in the case of two of the fungi tested. Aluminum is more toxic than gallium or indium, but less so than thallium.

Discussion. Although boric acid in the present tests in dilute solution did not prove toxic, borax in much greater concentrations has been developed as a dip to prevent the moulding of citrus fruits by *Penicillium* spp. and other fungi (25). Aluminum and thallium compounds have had little application as fungicides. A colloidal aluminum compound proved ineffective against the grape *Peronospora* in Italy (9) and conflicting results have been reported as to the efficacy of alum sprays against the same disease in France (67, 71). However, aluminum sulphate has been shown to be a most efficient soil disinfectant for *Rhizoctonia* damping-off of conifers (77). Burström (11) reported thallium the most toxic of the elements he tested. Thallium carbonate has shown distinct promise in the control of mildew on cotton goods (55). Further study may determine other fungicidal uses for thallium compounds.

CARBON, SILICON, GERMANIUM, TIN, AND LEAD

With this group we enter that part of the periodic table in which lie the elements which form simple volatile hydrides. These hydrides have been studied particularly by Paneth (61) who presents a review of the subject. The present authors have found that sulphur exerts its toxic action because the fungous spores have the property of reducing it to the hydride, H_2S (47). A number of other hydrides such as H_2Se , PH_3 , AsH_3 , SbH_3 have also been found extremely toxic to fungous spores. It is possible that the same mechanism of toxicity may apply to other hydride-forming elements besides sulphur. However, elementary selenium and tellurium were found to be non-toxic, although the hydride of selenium, H_2Se , is extremely toxic (79). It is a characteristic of these hydride-forming groups that the toxicity depends on the state of combination of the element. For example, sulphates are non-toxic, sulphites slightly toxic, while H_2S is extremely so. The other elements lying to the left of the carbon group in the periodic table do not exhibit such differences. Thus chromium is toxic as $KCrO_4$, or as chrome alum, although the valence of the element is different in these two compounds. For this reason it is difficult to assign a specific toxicity to the element, in the case of these hydride-forming elements. In presenting the results of the experiments in Figures 2 to 5 the toxicity of the hydrides has not been considered in assigning values to the elements.

A sample of elementary powdered silicon when dusted on glass slides was found to be toxic. This same powder, however, after being treated with HCl and subsequently washed, was non-toxic. It seems probable that

the sample contained silicides which on decomposition would give rise to silicon hydrides. Such compounds would probably be toxic, since all the volatile hydrides of the elements which have been tested are toxic. Na_2SiF_6 was non-toxic. Germanium dioxide, GeO_2 , was not appreciably toxic. Powdered tin, applied as a dust, was non-toxic, but powdered lead was highly toxic, as were also lead chloride, PbCl_2 , and acetate, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$.

Discussion. These results suggest that lead salts may have possibilities as fungicides. They have been reported as successful seed disinfectants (68).

NITROGEN, PHOSPHORUS, ARSENIC, ANTIMONY, AND BISMUTH

Phosphorus in the form of elementary red phosphorus was scarcely toxic. Arsenic, however, was toxic as arsenous acid, and as sodium arsenate, $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$. A sample of elementary powdered arsenic was also toxic. Antimony as potassium antimonyl tartrate, $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6 \cdot \frac{1}{2}\text{H}_2\text{O}$, and as metallic antimony dust was not toxic. Bismuth as powdered metal, and as bismuth ammonium citrate was also non-toxic.

Discussion. Although arsenic is not usually considered an active fungicidal element there are a number of references in the literature to the specific use of arsenical compounds as fungicides. For example, various American investigators have shown that arsenate of lead alone is somewhat effective in the control of apple scab, and that when added to lime sulphur it increases the fungicidal value of the latter (12, 33, 56, 57, 58, 59, 74). It has been found in England that certain arsenical compounds are definitely fungicidal toward *Sphaerotheca humuli* (30, 31, 36). *Alternaria panax* has been shown in New York to be more sensitive to calcium arsenate than to Bordeaux mixture (76), while in Italy *A. tenuis* and *A. fumaginoides* were very sensitive to sodium arsenite (26). Arsenical compounds are being used successfully as the only chemical means of controlling the apoplexy or esca disease of the vine in France and other Mediterranean countries (73) and also in California for "Black Measles" which is probably the same disease (8). Powdery mildew of cucumbers was controlled most satisfactorily in Russia with a sodium arsenate spray (69). A number of different arsenical compounds have been used as wood preservatives and these compounds have been considered as possessing the greatest fungicidal value among wood preservatives (24). As a result of the extensive investigations of Curtin and his associates zinc meta-arsenite has been developed as a commercial wood preservative (16, 17). In Russia (1, 7, 19, 21) and in Germany (40, 62) various arsenical compounds have been tested extensively and have given promising results as cereal seed disinfectants, especially for bunt of wheat. The results of Wollenweber (83) and of the present authors indicate a great variation in sensitivity to

arsenic among the fungi. Hence, like chromium, the practical application of arsenic will be confined probably to certain specific cases such as are noted above.

OXYGEN, SULPHUR, SELENIUM, TELLURIUM, AND POLONIUM

The three elements sulphur, selenium, and tellurium have been dealt with in previous publications from this laboratory (47, 78, 79), in which evidence is presented that the toxicity of sulphur is due to the fact that it exhibits an appreciable vapor pressure, and is readily reduced to H_2S by living material.

THE HALOGENS

The halides, NaF , $NaCl$, $NaBr$, NaI , as well as the oxidized forms, $KClO_3$, $KClO_4$, $NaBrO_3$, $NaIO_3$, $NaIO_4$, were non-toxic at concentrations under one millimol per liter.

Discussion. Sodium fluoride has been used somewhat extensively as a wood preservative and is the active principle of a number of commercial preparations (60), while sodium silicofluoride in experimental tests has given promising results in controlling bacterial leaf spot of the peach (2). It is well known that hypochlorites are toxic to fungous spores and are commonly employed as disinfectants (81). An aqueous bromine solution has been used successfully as a disinfectant for *Helianthus*, *Phalaris*, *Lobelia*, and *Mimulus* (63), and although iodine dust has been tried as a disinfectant for oat smut, there is no general agreement as to its effectiveness (29, 34, 39, 66, 75, 85).

The behavior of the halogen compounds is an excellent example of the fact mentioned previously, that the more negative elements exhibit wide differences in toxicity, depending on the particular type of compound tested.

GENERAL DISCUSSION

It might be anticipated that the fungicidal action of the elements would show a periodic relationship similar to that which is observed in the case of so many other physical and chemical properties. If we study Figures 2 to 5 it may be seen that this is true only to a limited extent. As a rule the toxicity within a group increases with increase in atomic weight, but there are notable exceptions. Thus gold appears less toxic than silver in each of the four cases studied. Chromium in two cases was more toxic than molybdenum or tungsten. The rare earth elements, so far as they have been tested, show a similarity in toxicity that might be expected from their chemical properties. It appears that the elements showing the highest toxicity lie in the center of the table, as presented here, and that the toxicity shows a tendency to decline toward the ends.

A question which presents itself is the extent to which the order of

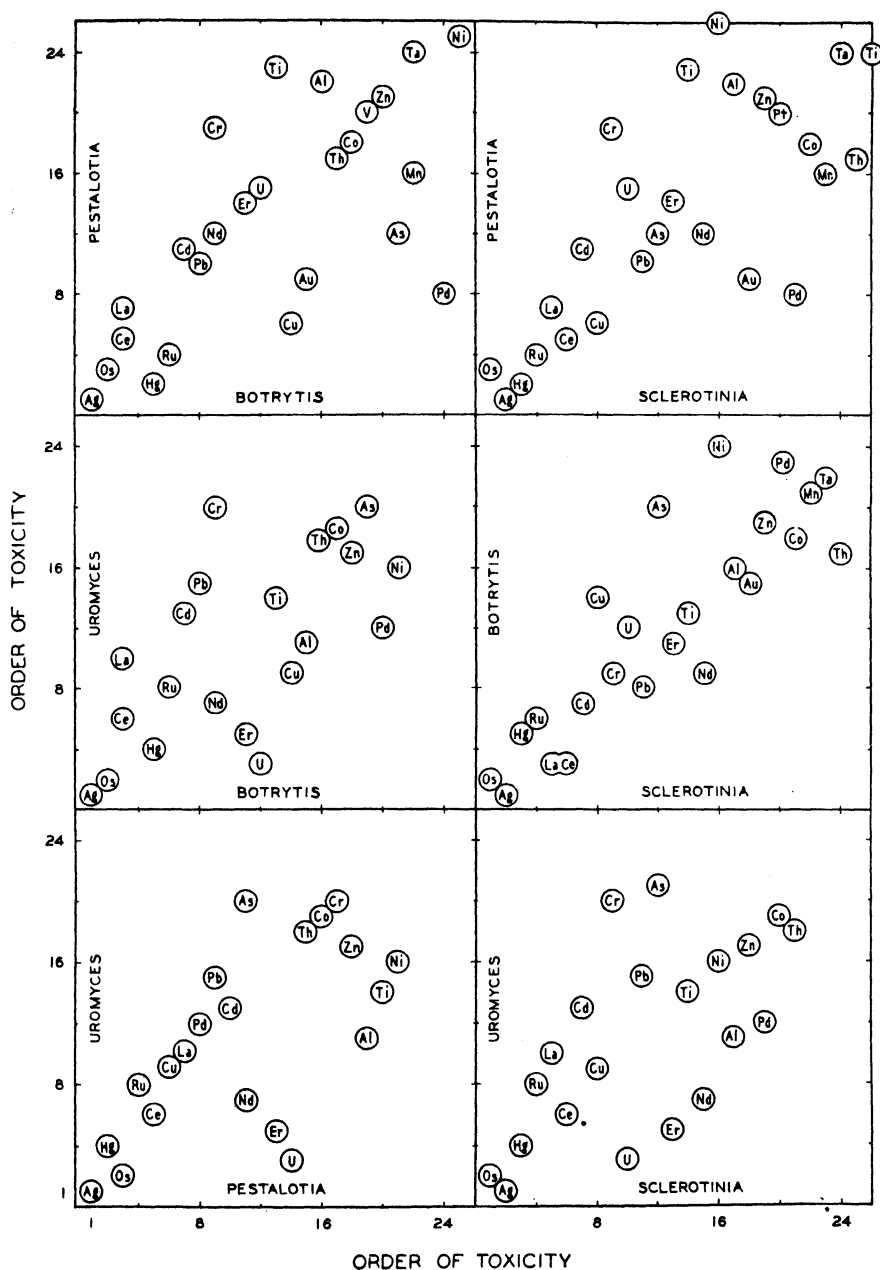


FIGURE 7. Correlation diagrams for the order of toxicity of the different elements with the four fungi, *Sclerotinia americana*, *Botrytis paeoniae*, *Pestalotia stellata*, and *Uromyces caryophyllinus*.

toxicity of the elements varies in the case of the four fungi used. If the elements are arranged in order of toxicity for the four fungi, correlation diagrams may be plotted as in Figure 7. Inspection of these diagrams shows that there is a marked tendency for a given element to lie in the same toxicity class for the various fungi. In other words, an element which is highly toxic to one fungus is likely to be toxic to the others. The correlation is highest in the case of the pair which are most closely related generically, *Sclerotinia americana* and *Botrytis paeoniae*.

SUMMARY

1. A study has been made of the toxicity of compounds of the elements to the conidia of *Sclerotinia americana*, *Botrytis paeoniae*, *Pestalotia stellata*, and to the uredospores of *Uromyces caryophyllinus* as related to the positions of the elements in the periodic system. The measure of toxicity employed was the concentration of the reagent permitting the germination of 50 per cent of the spores (LD 50).

2. In general the toxicity within a group increases with increasing atomic weight. The toxicity increases toward the center of the periodic table, as here presented, and is less at either end.

3. Compounds of the more positive elements showed practically the same toxicity regardless of the particular compound used, but in the case of the more negative elements the toxicity varied greatly with the particular type of compound used. The volatile hydrides so far as tested are all highly toxic, while the most highly oxidized forms show little or no toxicity.

4. There is a considerable tendency for an element which is toxic for one fungus to be toxic for others also, but the correlation is by no means perfect and many exceptions may be observed.

5. A number of elements were found to furnish compounds more toxic than those of copper. Compounds of silver and osmium were the most toxic. In addition to mercury and copper, toxic elements which may find more extensive application as fungicides are cerium, cadmium, lead, thallium, chromium, and arsenic.

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